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Controlling *Soil-borne cereal mosaic virus* in the UK by developing resistant wheat cultivars

by

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ABSTRACT

Soil-borne cereal mosaic virus (SBCMV) was detected in the UK for the first time in 1999. The virus, which is transmitted by the soil-borne protist *Polymyxa graminis* can cause serious yield losses in susceptible wheat cultivars. SBCMV is able to survive in the soil in the resting spores of *P. graminis* in the absence of wheat plants for at least 15 years. The development of resistant wheat cultivars for use in the UK is therefore essential if high yielding wheat cultivation is to be maintained on infested land.

The main objective was to identify genes involved in SBCMV resistance in wheat and develop molecular marker tags so that plant breeders can incorporate these genes in their breeding material. Resistance segregating in three populations derived from crosses between SBCMV resistant and susceptible parents was mapped using genome-wide QTL scanning. Two major resistances were identified, *Sbm1*, on chromosome 5DL and *Sbm2*, on chromosome 2BS. Lines carrying both genes showed significantly lower virus levels than lines carrying either gene alone, implying that the two resistances act in distinct and complementary ways to limit viral spread. AFLP markers closely linked to two resistance genes were identified for use in marker assisted selection.

An accelerated test for screening lines for SBCMV resistance was developed, using contained automatic immersion systems in controlled environments. Optimum conditions for virus multiplication were temperatures of 12-15°C with 2 hours watering in every 12 hours and material harvesting after 8 weeks. Testing root material for virus gave a better indication of resistance than testing leaf material. The test produced results which were very highly correlated with conventional field tests.

Representational Difference Analysis (RDA) was used to try to identify biosynthetic pathways activated during the onset of a resistance reaction. This knowledge could inform about the mechanism of resistance to SBCMV and contribute to an understanding of the likely durability of resistance. Results indicated that resistance mechanisms were activated as early as 12 days after introduction of the inoculum. A difference product was identified that increased only in resistant lines, indicating the involvement of the gene in the resistance mechanism.

Tests of the resistance of UK cultivars to a number of other closely related soil-borne viruses indicated that cultivars resistant to SBCMV are likely also to be resistant to soil-borne wheat mosaic virus (SBWMV) and vice-versa. In contrast, resistance to SBCMV and wheat spindle streak mosaic virus (WSSMV) do not appear to be related.

SUMMARY

INTRODUCTION

Soil-borne cereal mosaic virus (SBCMV) was detected in the UK for the first time in 1999, with 11 further cases being confirmed between 2000 and 2006. As the virus is no longer under statutory control in the UK it is likely that other cases have gone unreported. The virus occurs commonly in the USA, Egypt, China, Japan, Argentina, Brazil, Zambia, Italy, Germany and France and causes serious yield losses in susceptible wheat cultivars wherever it is found.

Symptoms of SBCMV infection in wheat vary from pale green to prominent yellow streaks on the leaves and leaf sheaths, accompanied by moderate to severe stunting. The virus is transmitted by the soil-borne protist *Polymyxa graminis*, and survives in the absence of host plants in the resting spores of *P. graminis*, which can remain viable in the soil for at least 15 years. Spread of the disease is by the movement of any soil infested with *P. graminis* that contains SBCMV particles.

Once land is infected by SBCMV, the only practicable means of control is to grow resistant cultivars. Screening of UK varieties on infected land has shown that a few UK cultivars are resistant, but these give growers only limited agronomic choice.

The development of resistant wheat cultivars for use in the UK is essential if high yielding wheat cultivation is to be maintained on infested land. The successful breeding of resistant cultivars is dependent upon the identification of resistant germplasm, the detection of genes conferring that resistance and the development of molecular markers to allow the inheritance of these genes to be monitored during accelerated marker-aided breeding programmes.

Some indication of possible sources of resistance in current cultivars can be gleaned from their pedigrees. The resistant cultivars Claire, Charger, Hereward, Cadenza and Flame appear widely in the pedigrees of recent resistant candidate cultivars. Evidence from elsewhere has implicated Moulin and the French cultivar Pernel as other possible resistance sources. Whether resistance in these cultivars is based on the same, or different, resistance genes is not known. Neither is there any evidence to indicate whether the resistance is governed by a single gene or by multiple genes. Recently it has been reported that the resistance of the cultivar Cadenza to SBCMV in artificially inoculated tests in the glasshouse could be explained by a single major gene. It is crucial that genetic studies are undertaken to investigate field resistance in contemporary cultivars.

The main objective of this project was to identify key genes involved in SBCMV resistance in wheat and develop molecular marker tags for their manipulation by breeders.

Other objectives were:

- to develop a quick, quantitative, accurate and reliable method for screening wheat cultivars for resistance to SBCMV in the glasshouse
- to investigate the mechanism of resistance to SBCMV in UK winter wheat cultivars
- to explore the possibility that resistance to SBCMV also confers resistance to other related soil-borne viruses

GENETIC MAPPING OF SBCMV RESISTANCE GENES

Experimental Approaches

Resistance observed to segregate in the Claire x Malacca (CxM) RILs populations and in the Avalon x Cadenza (AxC) DH population was mapped using a genome-wide QTL scanning strategy. From a set of almost 500 SSR primer pairs of known genomic location screened on the two populations, 121 and 107 polymorphic SSRs were mapped CxM and AxC populations respectively. To this skeleton framework of mapped markers, some 57 and 105 AFLP markers were added to boost genome coverage. In the case of AxC some data on DArT polymorphisms was made available through WGIN. Single marker analysis and Interval Mapping was performed using QTL Cartographer and R/QTL mapping software was also used to detect interactions between QTL.

Once major QTL had been located in particular genomic regions, Bulked Segregant Analysis (BSA) was used to identify closely linked AFLPs in *Sbm1* and *Sbm2* regions.

The opportunity arose as the project progressed to include a third population – Xi19 x Solstice (XxS) - in which the *Sbm2* resistance was separately segregating, and a limited set of phenotypic data and marker scores for chromosome 2BS were obtained for this population.

Results and Discussion

QTL Mapping of Sbm1 and Sbm2 and Interactions between the two loci

In the CxM population, the inheritance ratio of 1:1 indicated potentially a single major gene segregating. QTL analysis confirmed this with a single QTL of large effect on chromosome 5DL and a smaller effect but still statistically significant QTL on chromosome 2DL. In the AxC, the inheritance ratio was more complex and QTL analysis revealed two significant major QTL with LOD scores of

15 and 7 on chromosome 5DL and 2BS respectively. The 2BS resistance was more environmentally influenced than the 5DL resistance, however either locus alone was capable of conferring resistance in the field. The same closely linked AFLP marker was found under the 5DL QTL peak for resistance in both AxC and CxM populations and so this resistance was designated *Sbm1* following the published nomenclature for the gene from the Cadenza background. However, in contrast to published work on the AxC population which related segregation of resistance as measured by an artificial glasshouse inoculation method, we observed a second major resistance on 2BS expressed equally well in our CE and field evaluations.

This second gene, designated *Sbm2*, was of interest because a test for interactions showed an additive effect on resistance, that is DH lines of the AxC population carrying both genes showed significantly lower virus levels than those of lines carrying either gene alone. This means that the two resistances act in distinct and complementary ways to limit viral spread and proliferation, and suggests that pyramiding of the two genes as found in the cultivar Cadenza will be a useful strategy to prevent 'leakage' where environmental effects compromise individual resistance mechanisms and as a more effective barrier to the evolution of resistance-breaking isolates as has occurred with barley furoviruses.

In order to observe the segregation of *Sbm2* in the absence of *Sbm1*, a variety which had inherited *Sbm2* but not *Sbm1* from Cadenza was sought. Xi19 had been previously observed to have an intermediate reaction to SBCMV in the field and was therefore considered to be a likely candidate to possess the more environmentally influenced Sbm2 resistance. A DH population of Xi19 x Solstice (XxS) was evaluated in the field in one year and markers from chromosome 2BS used to construct a linkage map of that chromosome, which confirmed that *Sbm2* is the major gene resistance segregating in the Xi19 background.

Pedigree and chromosomal haplotype relationships between sources of resistance

Although not an objective of this project, it was of interest to understand how widespread resistance was in adapted winter wheats relevant to UK breeding, and to what extent this newly discovered *Sbm1* and *Sbm2* loci were shared amongst resistant varieties.

About 15% of historic UK and EU winter wheat varieties we have evaluated to date show resistance to SBCMV in the field and a significant proportion of these fall into three major pedigree lineages. The largest number of inter-related resistant varieties is accounted for by a lineage that traces back to early varieties Holdfast and Maris Widgeon. Moulin, Tremie, Flame, Genesis, Prophet, Bounty, Hereward, Exsept, Claire, Charger, Spark, Woburn and Goodwood all belong to this lineage and except for Holdfast, share the same uncommon allele of wmc765 which is the SSR marker closest to the peak of the *Sbm1* QTL. The second lineage including varieties Axona, Cadenza, Xi19,

Scorpion25, Aardvark and Cordiale most likely contains a mixture of Sbm1 and Sbm2 resistance and marker studies will clarify this situation. What our preliminary work indicates is that the *Sbm1* allele found in Cadenza is not tagged by the same 192bp allele of wmc765 which is diagnostic in the Holdfast-derived lineage.

A third lineage involves French-derived varieties Bersee, Elite Lepeuple and Flinor, so this and other apparently unrelated varieties showing field resistance may be sources of additional resistance genes and/or additional alleles of *Sbm1* and *Sbm2* which may be useful for the future protection of the wheat crop against SBCMV.

Closely linked markers to assist molecular breeding of resistance to SBCMV

The results of the mapping work have provided AFLP markers probably within 1 cM of both major resistances identified here, and a choice of genetically characterized elite sources of resistance have also been furnished. Feedback from the breeders in the project suggest that most breeding effort will be focused on introgression of *Sbm1*, as this resistance is currently effective on its own, and it is conveniently tagged by a diagnostic allele of SSR marker wmc765 when varieties related to Claire are used as sources of the gene.

DEVELOMENT OF A HYDROPONICS METHOD FOR SCREENING CULTIVARS FOR RESISTANCE TO SBCMV

Screening wheat lines for field resistance to SBCMV is a lengthy process requiring a full 8-month season. At the outset of the project CSL had already developed a re-circulating watering system to produce SBCMV infected plants to investigate the virus host range. The main objective of this study was to further develop and validate a method based on these contained automatic immersion systems for the rapid screening of SBCMV resistance in winter wheat lines. The validated method should mimic results obtained using conventional field studies.

Provisional experiments focused on finding the optimum temperature, growing period and watering regime to promote rapid SBCMV amplification. The results suggested temperatures of 12-15°C with 2 hours watering in every 12 hours and material harvesting after 8 weeks ideal for the virus multiplication. In addition, early experiments demonstrated testing root material gave a better indication of resistance behaviour than testing leaf material. The optimised controlled environment phenotyping test was validated against 18 cultivars of winter wheat with a known field reaction to SBCMV, as demonstrated in replicate field plots planted on land containing a natural inoculum source

of SBCMV. The optimised test correctly attributed a resistance rating for all the cultivars tested. Therefore the objective of creating an accelerated phenotyping test was completed.

The optimised phenotyping test was used to screen 3 double haploid (DH) populations were. The first was a publicly available DH population, derived from a cross between Avalon (susceptible) and Cadenza (resistant), consisting of 203 lines. The second was a DH population consisting of 198 individuals from a cross between Claire (resistant) and Malacca (susceptible) supplied by Nickerson-Advanta UK Ltd. The third population consisted of 113 DH lines derived from a cross between Xi19 (resistant) and Solstice (susceptible) supplied by Nickerson-Advanta UK Ltd. At least 14 replicates of each parental line were included in each experiment to act as controls. Experiments on DH populations AxC and XxS were repeated twice and lines from CxM were repeated three times. The field resistance to SBCMV of all DH populations was determined by growing each line in replicate field plots. Data were collected on virus symptoms and the leaf material tested for the presence of SBCMV using a specific immunological assay. Incidence and Severity visual scores were very highly correlated ($R^2 = 0.9188$), as too were ELISA values from either field or hydroponic tests ($R^2 = 0.837$).

The observed segregation ratio of resistant (R) and susceptible (S) lines for each DH population calculated based on the consensus phenotype. When all measures of SBCMV were considered together, chi-squared analysis showed that both the CxM and XxS mapping populations exhibited R:S ratios consistent with 1:1 segregation indicative of single Mendelian genes, while the AxC population (with Cadenza as resistance donor) showed a R:S ratio consistent with either 2:1 or 3:1 segregation suggesting the presence of more than one independent gene.

The results suggest there are possible 2 genes operating within the AxC population, not one as previously suggested. Furthermore, it appears that the rapid phenotyping test developed is a closer match of the field conditions than previously developed protocols demonstrating the importance of including comparative data from field trials to validate any potential screening method based in the laboratory.

INVESTIGATION OF THE MECHANISM OF RESISTANCE TO SBCMV IN UK CULTIVARS USING REPRESENTATIONAL DIFFERENCE ANALYSIS

Representational Difference Analysis (RDA) uses the technique of subtractive hybridisation to allow direct comparison of two populations of mRNA and enriches sequences that more highly expressed in one population than in the other. Essentially the method was used to try to identify which biosynthetic pathways were activated during the onset of a resistance reaction. This knowledge could inform

about the mechanism of resistance to SBCMV and therefore aid our understanding of the durability of such resistance mechanisms. This approach requires two populations of mRNA, for this two cultivars of wheat were chosen that are very closely related, cv. Claire (resistant) and cv. Istabraq (susceptible). In this study the gene expression for both cultivars was compared during infection with SBCMV to try and isolate gene fragments involved in the SBCMV resistance reaction.

A provisional time-course experiment suggested that SBCMV was present in a higher titre in Istabraq roots than in Claire roots after only 12 days, indicating the resistance mechanisms were activated at this early stage. Total RNA extractions were prepared from root samples taken 12 days after infection with SBCMV for cultivars Claire and Istabraq. The RDA method was carried out in both forward and reverse directions to sift for genes that were both up- and down-regulated in Claire compared to Istabraq. These difference products were then cloned, sequenced and then compared to all known protein and nucleotide sequences using a combination of searches from the NCBI and TIGR databases. In total 56 clones from the forward subtracted and 16 clones from the reverse subtracted libraries. Of 47 clones sequenced, 6 produced no similarity with any known sequence. Sequences of the expressed difference products showed similarity to a range of proteins from barley, rice and wheat including: mRNA-gibberellin responsive gene; aquaporin; fragments from rice chromosome 2; and intracellular signalling proteins.

Real-time PCR assays were designed to two difference products, C sub A9 (similar to gibberellin responsive genes) and C sub G1 (analogous to a short section on chromosome 2 of rice). A time-course experiment was set up using Claire and Istabraq and five each of resistant and susceptible isogenic lines from the Avalon x Cadenza population. All lines were planted in soil containing SBCMV and soil free from SBCMV. The C sub A9 and C sub G1 real-time assays were used to quantify the amount of product present in nucleic acid extracts prepared from triplicate samples taken at 5, 7, 9, 12, and 14 days after planting and the results normalised against an RNA specific control. These results demonstrated C sub A9 increased in expression after 14 days in both uninfected and infected soil, suggesting C sub A9 is not involved in SBCMV resistance. The levels of C sub G1 also rose after 14 days but only in resistant isogenic lines and Claire in infected soil suggesting this gene is probably involved in the SBCMV resistance mechanism. This section of the project produced many other sequences that could yield very interesting results. A great deal of resources would be required to further investigate all of the sequences isolated by RDA, however this study has demonstrated that RDA is a powerful tool to identify genes more highly expressed in one mRNA population than in another, and has proved itself to be a useful method to identify sequences that may be involved in the mechanisms of resistance to SBCMV in wheat. Other technologies (e.g. microarrrays) emerged during the project, and these would also be suitable for use in such a study.

INVESTIGATION OF RESISTANCE OF UK CULTIVARS TO OTHER RELATED SOIL-BORNE VIRUSES

The aim of this part of the study was to explore the possibility that resistance to SBCMV also confers resistance to other related soil-borne viruses including *Soil-borne wheat mosaic virus* (SBWMV) (USA), *Wheat spindle streak mosaic virus* (WSSMV) (USA), *Aubian wheat mosaic virus* (AWMV) (France). In total 13 cultivars were selected with a known resistance reaction to at least one virus. Soil was collected from three fields, the first known to contain AWMV in France, the second SBCMV from the UK and the third a mixture of SBWMV with WSSMV collected from the USA. Each soil was diluted 50:50 with sterile sand separate apparatus automatic immersion systems as developed for rapid phenotype screening for SBCMV resistance. All the experiments were conducted at 15°C within a controlled environment room for 14 weeks prior to root and leaf material being harvested and tested for the presence of the respective virus using specific immunological protocols for each virus.

Good data were obtained for 10 of the 13 cultivars tested in soil containing SBCMV, SBWMV and WSSMV. However, despite growing the wheat plants in ideal conditions for the propagation of AWMV, all the plants grown in soil supposedly containing AWMV tested negative for this virus. Known AWMV susceptible cultivars were included in the trial and these consistently tested negative for the virus. These results suggest infectious AWMV was absent from the soil sample sent from France.

Similar levels of virus were recorded in leaf and root material for cultivars known to be susceptible or resistant to WSSMV. This suggests the method used to test AWMV, SBCMV and SBWMV was not suitable for encouraging WSSMV. Therefore it can be concluded that the resistance mechanisms for these two viruses are likely to act in different ways.

Cultivars known to be susceptible to SBCMV or SBWMV had consistently higher levels of virus in root material than cultivars known to be resistant to either virus. Therefore a cultivar known to be resistant to SBCMV is likely to also be resistant to SBWMV and visa-versa. These data suggest the resistance mechanisms are similar for both viruses and publications offering insight into the biology of SBWMV or looking at cultivar resistance could provide useful information for studying SBCMV. For example, two resistance genes were identified in Brazilian cultivars of *Triticum aestivum* using screening of backcross populations derived from resistant and susceptible parents.

INTRODUCTION

Soil-borne cereal mosaic virus (SBCMV) was detected in the UK for the first time in 1999, on a farm in Wiltshire. Between 2000 and 2006, 11 further cases have been confirmed, mainly in Kent and Wiltshire but with occasional other reports from the south of England. However, as the virus is no longer under statutory control in the UK it is likely that other cases have gone unreported. The virus occurs commonly in the USA, Egypt, China, Japan, Argentina, Brazil, Zambia, Italy, Germany and France. In France, it was first reported in 1978 and is now widely distributed in central and western regions and is rapidly spreading northwards and eastwards. SBCMV causes serious yield losses in susceptible cultivars of winter wheat wherever it is found. Trials at the infected site in Wiltshire showed that the virus can reduce grain yield in susceptible cultivars of winter wheat by up to 50% (Clover *et al.*, 2001a, Budge *et al*, 2002).

Symptoms of SBCMV infection in wheat vary from pale green to prominent yellow streaks on the leaves and leaf sheaths, accompanied by moderate to severe stunting. Symptoms are most obvious on early spring growth. As new leaves unfold they appear mottled and develop parallel dashes and streaks. Fields usually show a patchy incidence of the disease. SBCMV is transmitted by the soil-borne protist *Polymyxa graminis*, as are the soil-borne viruses of barley, *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV). The virus survives in the absence of host plants in the resting spores of *P. graminis*, which can remain viable in the soil for at least 15 years and can also survive in manure from animals fed on contaminated feedstuff. Spread of the disease is by the movement of any soil infested with *P. graminis* that contains SBCMV particles. Movement of soil may be by farm vehicles and machinery, footwear, livestock, wind blow and surface water. There is some evidence to suggest that SBCMV may be seed-borne in rye, however it is thought that the most likely route of entry into the UK must have been as a contaminant of soil associated with seed potatoes, root vegetables, nursery stock and other transplants. Experience of the spread of SBCMV in other countries, together with UK experience of the soil-borne mosaic viruses of barley, indicates that there is a high risk that SBCMV will become widespread in the UK, particularly as most of the wheat cultivars used in the UK prior to 1999 were susceptible to the virus.

Once land is infected by SBCMV, the only practicable means of control is to grow resistant cultivars. Control of the fungal vector by fungicides or soil sterilisation is ineffective and uneconomic. In France, cultivars with good resistance have been identified (e.g. Fandango, Tremie, Taldor) and breeding work is in progress to produce improved cultivars for the future. Cultivars from the UK recommended list have been screened for resistance to SBCMV and another soil-borne virus (*Wheat spindle streak virus* (WSSMV)) using infected sites in France and Italy (CSL HGCA project no

2182). Results from this project have shown that the majority of the UK cultivars became heavily infected with SBCMV. Typical symptoms, including leaf streaking and stunting, were seen on all cultivars with the exception of Charger, Claire and Hereward. Low levels of foliar symptoms were seen on cultivars Aardvark and Cockpit. During the experiment, Aardvark, Cockpit, Charger, Claire and Hereward showed little or no visual symptoms of SBCMV and their final yield seemed unaffected by the virus. It can be concluded that these cultivars are either resistant or partially resistant to the disease. All other cultivars tested during this project proved susceptible to SBCMV. Yields of the susceptible cultivars were reduced to as little as 40% of the average for the site. None of the UK winter wheat cultivars became infected with WSSMV when grown on infected land. This strongly indicates that all UK cultivars tested are immune to WSSMV. In the UK, more than 100 cultivars and NL candidates were screened for resistance on an infected site as part of another HGCA-funded project conducted by NIAB (Project 2254). Approximately 20% of these cultivars proved to be resistant to SBCMV. These results demonstrate the existence of resistance in the gene pool of UK cultivars. However, as only three out of the 29 cultivars on the UK Recommended List for 2006/2007 are resistant (with two others possibly resistant, but requiring further tests), farmers on infected land currently have very limited agronomic choice.

The development of resistant wheat cultivars for use in the UK is essential if high yielding wheat cultivation is to be maintained on infested land. The successful breeding of resistant cultivars is dependent upon the identification of resistant germplasm, the detection of genes conferring that resistance and the development of molecular markers to allow the inheritance of these genes to be monitored during accelerated marker-aided breeding programmes. The availability of resistant cultivars will place UK farming in a much stronger position to deal with the anticipated spread of SBCMV into major areas of the UK wheat crop.

Some indication of possible sources of resistance in current cultivars and NL candidates can be gleaned from their pedigrees. The resistant cultivars Claire, Charger, Hereward, Cadenza and Flame appear widely in the pedigrees of recent resistant candidate cultivars. Evidence from elsewhere has implicated Moulin and the French cultivar Pernel as other possible resistance sources. Whether resistance in these cultivars is based on the same, or different, resistance genes is not known. Neither is there any evidence to indicate whether the resistance is governed by a single gene or by multiple genes. Recent genetic studies indicated the presence of two genes controlling resistance to *Soil borne wheat mosaic virus* (SBWMV) in Brazilian wheat cultivars (Barbosa *et al.*, 2001). Earlier, Merkle & Smith (1983) concluded that reaction to SBWMV in certain US wheat cultivars was controlled by one, or two, dominant genes. Recently, Bass *et al*. 2006 reported that resistance of UK cultivar Cadenza to SBCMV in artificially inoculated tests in the glasshouse was governed by a single major gene mapping to the distal portion of 5DL, a location also identified by Narasimhamoorthy *et al*. 2006

as carrying a major QTL for field resistance to SBWMV. Given that effective tolerance to the virus under field conditions is relatively commonplace, it is crucial that genetic studies are undertaken to dissect this type of resistance in contemporary genotypes.

The main objective of this project was to identify key genes involved in SBCMV resistance in wheat and develop molecular marker tags for their manipulation by breeders.
Other objectives were:

- to develop a quick, quantitative, accurate and reliable method for screening wheat cultivars for resistance to SBCMV in the glasshouse
- to investigate the mechanism of resistance to SBCMV in UK winter wheat cultivars
- to explore the possibility that resistance to SBCMV also confers resistance to other related soil-borne viruses

The initial stages of marker development depend on phenotyping large numbers of lines from populations segregating for resistance and classifying each as susceptible or resistant. One approach to phenotyping is to grow mini plots on infected land and monitor natural infection in the lines. Although the use of mini plots on infected land is inexpensive and is generally satisfactory for assessing the resistance of cultivars to SBCMV (Bayles and Napier, 2002) there are drawbacks for genetic work. These include the risk of false 'negatives' due to patchy distribution of the virus over the test site, a relatively high risk of plot loss due to such factors as predation, unfavourable weather and weediness and the long period (probably 7-8 months) between sowing and final assessment of symptoms. A reliable and repeatable artificial test for resistance that can be carried out under controlled glasshouse or growth room conditions and deliver results for large numbers of individual plants within a short period was considered essential for the success of this project. Such a test could also have more general applications as a tool for breeders and others wishing to evaluate resistance to SBCMV.

The mechanisms of resistance to SBCMV are unclear. However, the lack of virus in leaf material of resistant cultivars suggests that resistance may act by reducing virus movement to the aerial parts. Driskel *et al.* (2002) recently concluded that virus resistance in hard red winter wheat probably operates in the roots to block virus movement to the leaves. As part of this project, the mechanism of resistance was investigated using Representational Difference Analysis (RDA), a technique which allows the enrichment of genes differentially expressed between two samples, in this case Resistant and Susceptible cultivars challenged by SBCMV. The aim was to clone and sequence fragments of genes switched on in the roots of resistant cultivars during infection by SBCMV. These should represent primary and secondary genes activated during infection and bioinformatics analysis of

homology to genes of known function would give indications as to the biochemical, developmental or signalling pathways involved.

Soil borne wheat mosaic virus (SBWMV) was first identified in the USA in 1919. Since then a range of similar viruses have been identified across Europe, China and Japan (Diao *et al* 1999; Koenig *et al* 1999;Clover *et al*., 2001b). The SBCMV group of viruses (which includes the UK Wiltshire isolate) have been identified as having some differences in biological behaviour when compared with the SBWMV group, for example, differences in reactions to indicator plants like *Chenopodium quinoa*. Also, nucleic acid sequences for RNAs 1 and 2 in the SBCMV group of isolates differ substantially from those of the US SBWMV isolates, showing only 70% (RNA1) and 66%(RNA2) homology. The SBCMV group of viruses has therefore now been renamed to distinguish them from SBWMV. Within the SBCMV group there appears to be considerable sequence variability, with the Chinese and Japanese isolates in particular showing significant differences from other isolates, but these have not so far been linked to biological differences. Other recent developments continue to complicate the picture. The presence of SBWMV was recently confirmed in Germany (Koenig & Huth, 2003), causing disease in wheat and a barley-infecting virus found in France was reported to be related to the US SBWMV by Hariri *et al* (2005). It was therefore considered important to investigate the reaction of wheat resistance genes to both virus species within this project.

All isolates of SBCMV were once thought to belong to the same species. However, Shirako *et al.* (2000) recently concluded that European, American, Chinese and Japanese strains of the virus can be distinguished from one another on the basis of their nucleic acid sequence. Strains of SBCMV are known to differ in various biological characteristics including host range (Brakke & Langenberg, 1987) but it is not clear whether this is related to the sequence differences identified. These strains may also differ in their interaction with plant resistance genes The UK Wiltshire SBCMV isolate has been sequenced and confirmed to be similar to the European strain of the virus (Clover *et al.*, 2001b). Two other soil borne viruses are known to infect winter wheat crops across Europe, *Wheat spindle streak mosaic virus* (WSSMV) and *Aubian wheat mosaic virus* (AWMV). Both viruses have been reported as causing considerable yield loss in France. Studies carried at by CSL using field trials in France & Italy have suggested that all UK winter wheat cultivars are resistant to WSSMV. Although superficially similar to SBCMV, AWMW is considered to be a distinct virus on the basis of serological differences. AWMV is also thought to be similar to a virus reported from Bedfordshire in 1995 (Clover *et al*., 1999). Wheat cultivars were checked not only for resistance to the European strain of SBCMV, but also to an isolate from the USA and to soil-borne viruses WSSMV and AWMV. This should indicate the extent of any relationship between resistance to the different strains and viruses.

GENETIC LINKAGE MAPPING OF SBCMV RESISTANCE GENES

Introduction

In order to locate the major genes segregating in the CxM and AxC populations, a QTL scanning approach was taken whereby genome-wide linkage maps were generated for both populations and used to detect QTL loci affecting reaction to SBCMV infection. Microsatellite markers or SSRs being the most informative and transportable class of marker were used to construct the backbone of each linkage map, and supplemented with AFLPs, DArT markers and AFLPs targeted to resistance loci by Bulked Segregant Analysis (BSA).

Materials and Methods

Microsatellites -

Publicy available microsatellites were obtained from a range of sources Roder *et al*. 1998, http://wheat.pw.usda.gov, www.scabusa.org) with the initial aim being to obtain reasonable genome coverage (20 –30 cM intervals) to allow linkage groups to be detected with the random AFLP markers and the resistance segregation data. The consensus maps published for wheat by Somers *et al*. 2004 were useful in designing this skeleton coverage. Amplification was conducted under standard protocol conditions, as indicated with the original published details, with one primer fluorescently labelled for use on ABI capillary electrophoresis equipment. Microsatellites were tested on the DNA from parent lines; polymorphic markers were then applied to the segregating populations. The AxC population was mapped with 99 microsatellites, while 127 microsatellites were mapped in CxM.

Genome-wide and targeted AFLP mapping

AFLP analysis followed a protocol modified from Vos *et al*. 1995. DNA samples (500ng) were digested with restriction enzymes (*Eco* RI and *Mse* I) for 1 hour prior to ligation of double-stranded adaptor sequences overnight (all at 37 °C). Pre-selective PCR amplification was then performed using a single base extension to the *Eco* RI and *Mse* I primer sequences (A and C respectively). Aliquots of these products were then used for selective amplification with a range of 3 base extensions to both primers, the *Eco* RI sequence being labelled with the fluorescent IRD 700 dye (MWG) suitable for visualisation on Li-Cor automated gel electrophoresis apparatus. Sample loading (0.5 µl) and electrophoresis conditions followed manufacturer's guidelines. Gel images were analysed visually for the presence / absence of segregating bands. Initial choice of primer combinations was made on the basis of maximising polymorphisms between the parents of the mapping populations, in order to generate a large number of markers on the chromosomes prior to initial mapping exercises. This involved 77 polymorphic bands for AxC, and 57 for CxM. Once the candidate regions and flanking markers had been identified, further AFLP screening was performed on bulk DNA samples comprised

of susceptible versus resistant segregating lines. Lines with a recombination between a flanking marker and the resistance genes were selected to comprise bulks of a minimum of 5 lines; in the population with 2 resistance genes, two resistant bulks were created, one for each gene, with the marker data indicating the susceptible allele was present at the alternative locus.

DArT

DArT marker segregation data for the AxC population was supplied by Michelle Leverington and John Snape (JIC) through mapping work taking place as part of the Defra Wheat Genetic Improvement Network (WGIN). We have included here only those DArT features which mapped to chromosomes 2B and 5D.

Map construction

Molecular marker data for the individual lines of the segregating populations was scored according to the parental profile following the convention of female parent alleles assigned as 'A' and male parent alleles as 'B'. This data was formatted for analysis using the JoinMap®3.0 programme (Kyazma B.V.). Mapping analysis was conducted using standard (default) programme settings

Statistical and QTL analysis

QTL analysis was performed in R-QTL (Broman *et al*. 2003) and QTL Cartographer (Wang *et al*. 2006) using marker orders and distances calculated using Joinmap.

Results

Genetic mapping of two major-effect QTL for SBCMV resistance

In the AxC population, on which a total of 107 polymorphic SSR markers , 105 AFLPs and 18 DArT markers were genotyped across lines 1-92, single marker ANOVA (Table 3) showed two very highly significant blocks of markers associated with resistance on chromosomes 5DL and 2BS and a smaller, yet significant, effect on chromosome 6AS. The QTL on 5DL (*Q.SbmCz1*) - corresponding to the *Sbm1* gene reported by Bass *et al*. 2006 - and 2BS (*Q.SbmCz2*) were confirmed by interval mapping LOD across all 21 linkage groups but in this case, no additional QTLs reached the threshold of significance (Figure 4).

Table 3 : Summary of significant single marker ANOVA associations with SBCMV reaction and Interval Mapping QTL intervals

Cross	Phenotype	Chromo-some	Marker	Pr(f)	Star	SMA Var expl	Threshold (1000 perms, 0.05)	MIM Var expl	MIM 2Bx5D Var expl	Effect	MIM peak cM	MIM peak LOD	
AxC	Elisa all	2BS	aflp100.175	0	****	27.0	4.52	25.20%	13.9 % (AA)		15.2	8.5	
AxC	Elisa all	5DL	AFLP347; AFLP24	0	****	45.1	4.52	44.70%			114.1	10.6	
AxC	Log all	2BS	aflp100.175	0	****	27.3		25.10%	7.8 % (AA)		16.1	7	*
AxC	Log all	5DL	AFLP347; AFLP24	0	****	49.9		42.20%			113.4	11.2	
AxC	Elisa Field	2BS	aflp100.175	0	****	16.8		10.70%	5.4 % (AA)		16.2	3.9	*
AxC	Elisa Field	5DL	AFLP347; AFLP24	0	****	49.0		43%			113.2	9.2	
AxC	Log Field	2BS	aflp100.175	0	****	15.8		13.10%	9.9 % (AA)		16.2	4.1	
AxC	Log Field	5DL	AFLP347; AFLP24	0	****	52.4		44%			113.2	9.1	
AxC	Elisa hyd	2BS	aflp100.175	0	****	34.0		33.20%	15.6 % (AA)		16.2	9.9	*
AxC	Elisa hyd	5DL	AFLP347; AFLP24	0	****	33.3		28.10%			113.1	10.2	
AxC	Log Hyd	2BS	aflp100.175	0	****	43.9		45.80%	5.9 % (AA)		16.7	9.4	
AxC	Log Hyd	5DL	AFLP347; AFLP24	0	****	33.1		26.40%			113.2	8.3	
AxC	Incidence	2BS	aflp100.175	0	****	24.7		22.90%	19.8 % (AA)		15.2	9.4	*
AxC	Incidence	5DL	AFLP347; AFLP24; AFLP57	0	****	37.7		30.90%			113.1	8.8	
AxC	Severity	1BL	eACTmCAC115	0.002	**	11.4		11.60%			not sig	not sig	
AxC	Severity	2BS	aflp100.175	0	****	21.3		17.20%	9.5 % (AA)		15.2	5.6	*
AxC	Severity	5DL	AFLP347; AFLP24	0	****	44.1		39.30%			113.1	8	

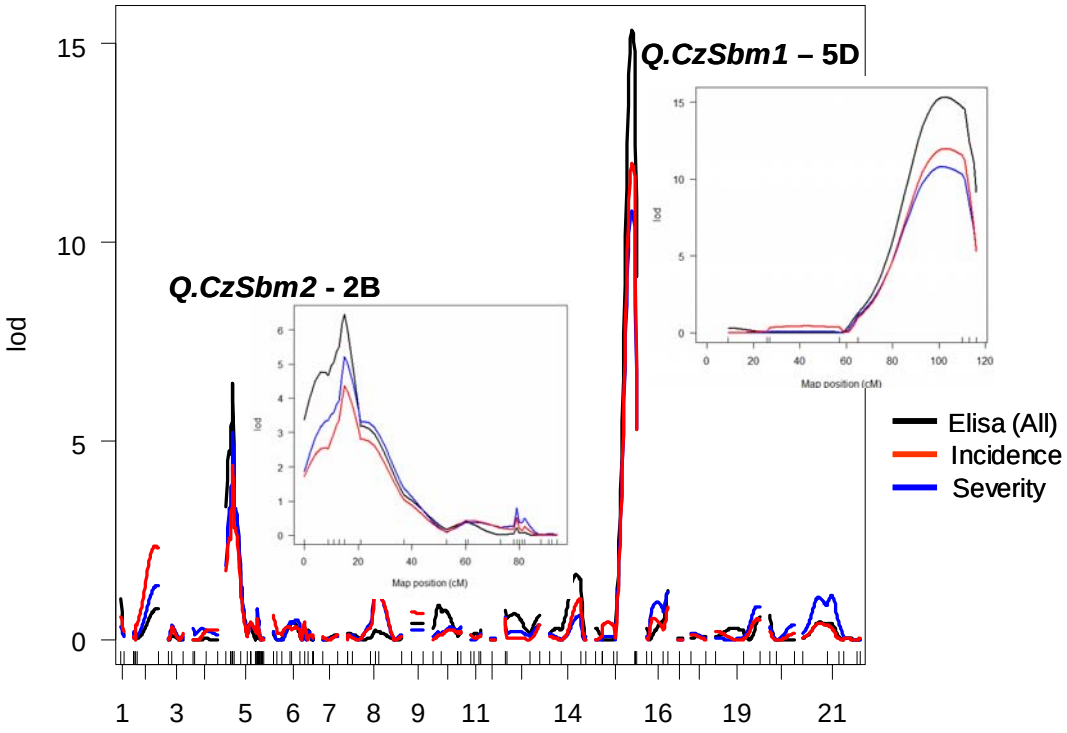


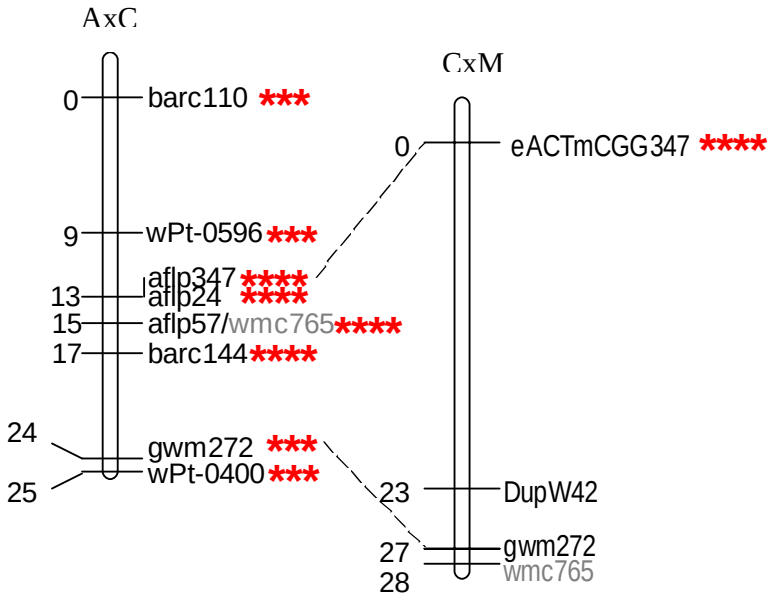
Figure 4: QTL interval mapping SBCMV resistance in AxC population.

In the CxM population, a total of 121 polymorphic SSR markers and 57 AFLPs were genotyped on lines 1-92. Single marker ANOVA identified a major QTL for resistance on 5DL (*Q.SbmCl1*) and a minor QTL on 2DL (Q.*SbmCl2*). Figure 5a shows the 5DL linkage groups generated by Joinmap for AxC and CxM populations respectively, showing the significance of association of each marker with the phenotype. These maps are essentially colinear and in both populations the marker nearest the peak LOD was AFLP347, with the marker in phase with resistance, therefore in the absence of genetic proof of allelism, we assume that either Cadenza and Claire share common ancestry which has resulted in both inheriting the same resistance allele, or that they carry allelic forms of a single locus, and so we will henceforth refer to these coincident *Sbm1*/*Q.SbmCz1*/*Q.SbmCl1* as *Sbm1*. In order to further investigate the nature of resistance mediated by the 2BS resistance locus (*Q.SbmCz2*), we took advantage of the availability of a third segregating population of 113 DH lines from a cross between Xi19 and Solstice (XxS), known from previous work to be resistant and susceptible respectively (Bayles and Napier 2002). Xi19 was assumed to carry one or other of the Cadenza genes, given its close pedigree relationship, therefore the markers from the major QTL regions identified above (5D &

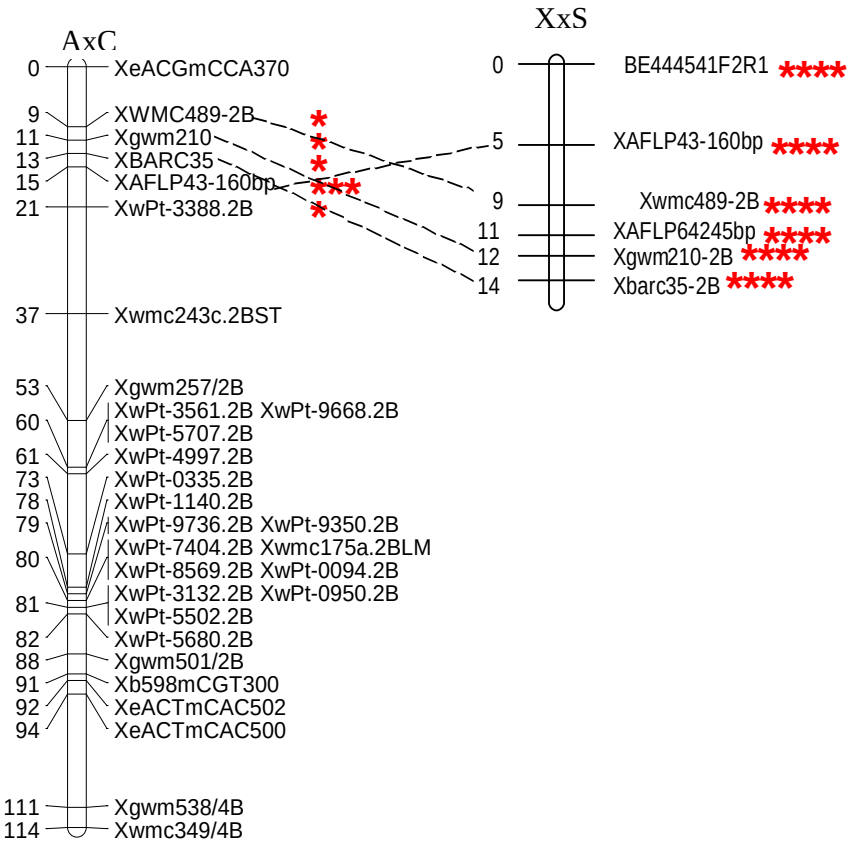
2B) were tested for polymorphism in the XxS population and mapped as single linkage groups without any additional genome coverage. Figure 5b shows the comparative 2B linkage map for AxC and XxS populations and again single marker ANOVA confirms that *Q.SbmCz2* and *Q.SbmXi1* both map to the short arm of 2B. We propose that these coincident major-effect QTL on chromosome 2BS be designated *Sbm2.* It is of some considerable interest to note that *Sbm2*-mediated resistance, although perfectly obvious in the field (absence of symptoms), is not expressed under conditions described by Kanyuka *et al*. 2004, indicating that it has a distinct mechanism of action to *Sbm1*.

Figure 5: (A) Comparative linkage maps of chromosome 5DL between AxC (left) and CxM (right) populations (B) Comparative linkage maps of 2BS between AxC (left) and XxS (right) populations. The significance of association between each marker and resistance to SBCMV as determined by single marker ANOVA analysis with Bonferroni correction is indicated by the red asterisks.

A.



B.



Gene interactions

The AxC population, segregating for both genes, allowed us to assess epistasis and interactions between *Sbm1* and *Sbm2*. A strong epistatic additive effect was observed calculating the LOD for epistasis between all pairwise combinations of loci in R-QTL (Figure 6a). Another way of viewing the interaction is provided in Figure 6b, whereby the phenotypic values of lines are grouped and plotted according to their bi-locus genotype or inferred genotype for the two major QTLs *Sbm1* and *Sbm2*. This shows that lines, which on the basis of their genotype are assumed to carry *Sbm1* or *Sbm2* resistance separately have average logELISA scores of -0.67 and -0.58 respectively (compared with -0.05 for lines carrying neither), while those lines inferred to carry both QTL have a significantly lower logELISA average score of -0.84. It is notable that while the distribution of phenotypic values for the *sbm1 Sbm2*, and to a lesser extent the *Sbm1 sbm2* class, appear to show some leakage whereby the values for a few individual lines overlap with the lower part of the susceptible genotype range, the lower average score for this class is due in part to the tighter clustering of values.

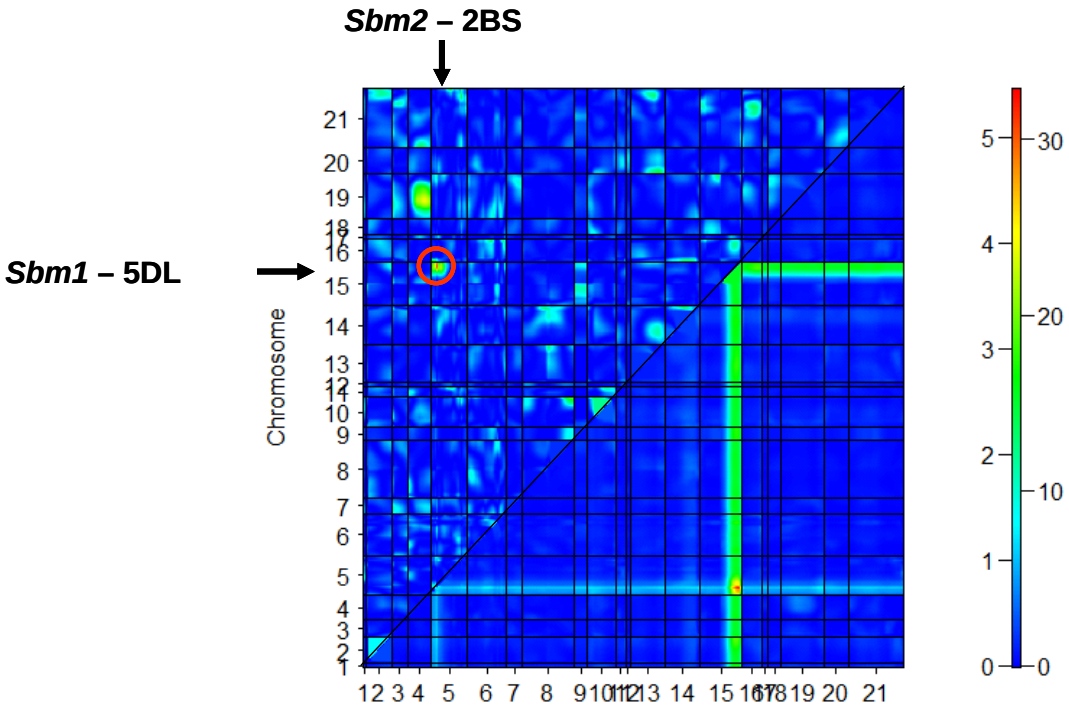
Thus we can conclude that the differing mechanisms of resistance offered by the respective genes are complementary and that in genotypic backgrounds or environmental conditions where one is weakly expressed, the expression of the other locus acts as a failsafe.

Potential mechanisms

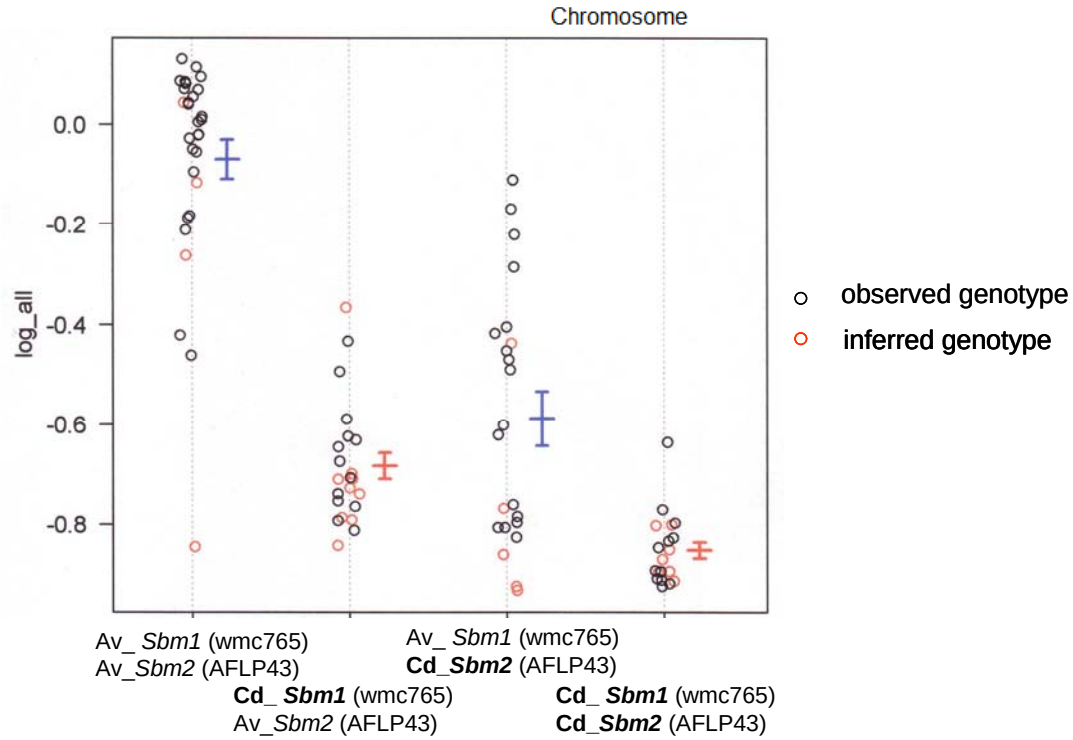
The comparison of the results obtained by Kanyuka *et al*. (2004) to those obtained in this study offers an insight into the possible resistance mechanisms of the two resistance genes identified in the A x C DH population. The *Sbm1* locus was detected both by Kanyuka *et al*. (2006) and in this current study, suggesting the mechanism of resistance is acting to limit either i) virus multiplication or ii) virus movement in the roots and also virus movement to the leaves. In contrast to the *Sbm1* locus, *Sbm2* was not detected by Kanyuka *et al*. (2004), suggesting a different mode of action restricted to limiting the build-up or movement of the virus in root tissue. The discovery of both loci in a single segregating population offers the possibility for adopting a gene pyramiding strategy, offering potentially valuable additive and more durable field-resistance.

Figure 6: (A) Heat map showing likelihood of epistatic interactions between all locus pairs across the 21 chromosomes for the AxC linkage map. Above the diagonal is the LOD for epistasis. Only the patch of bright colour circled in red is significant for epistasis between chr5 (chr2B) +chr15 (chr 5D) QTL is significant on a permutation test. Below the diagonal is the LOD for the pooled effects of (QTL 1 +QTL 2 + epistasis). (B) Phenotypic values of individual AxC DH lines grouped by genotype at marker locus under *Sbm1* and *Sbm2* QTL peaks. Red circles represent lines where the genotype at the QTL is inferred (in the case where flanking markers were missing or conflicted).

A.



B.

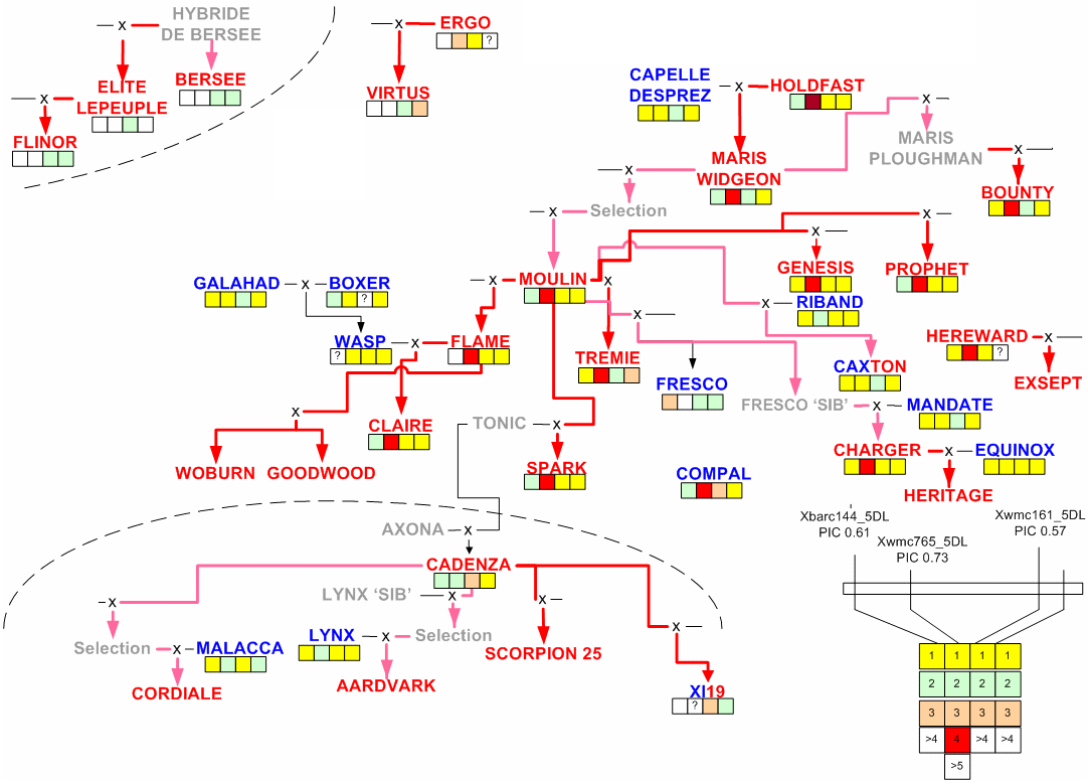


Pedigree and chromosomal haplotype relationships between sources of resistance

SBCMV was first noted in the UK in 1999 and all currently recommended commercial cultivars that carry resistance - Claire, Cordiale, Hereward and Xi19 - were bred and registered as cultivars prior to the confirmation of SBCMV in the UK, and therefore the availability of SBCMV-resistant cultivars bred for UK market must be viewed as being somewhat serendipitous. To complement our genetic analysis of SBCMV resistance in the principle sources among current cultivars, which showed two major loci to be present, we undertook further investigation of the origins and extent of SBCMV resistance in a wider set of 186 UK and European germplasm by surveying the SBCMV resistance and marker genotypes for the *Sbm1* and *Sbm2* regions of cultivars which were members of the extended pedigrees to which the main current sources of resistance belong. Our results are summarised in Figure 7. In total, we observed 11 instances of resistant cultivars in a Holdfast-derived lineage spanning four generations of cultivar development and six instances in an Axona-derived lineage spanning three generations. Two further clusters of resistance were observed: three old French varieties – Bersee, Elite Lepeuple and Flinor – and the parent progeny combination, Ergo and Virtus, which represent distinct pedigree lineages. In addition, a number of other instances of resistant varieties with no obvious pedigree relationship to any of the four groups outlined above. In total, 15% of varieties tested showed effective field resistance.

In order to investigate whether *Sbm1* marker haplotypes were conserved among any of these resistant lineages, which would suggest inheritance of a common source of resistance, four SSRs (as shown in Figure 7) spanning 10 cM around the QTL peak were scored on the full set of 186 varieties. Interestingly, only wmc765 was associated with inheritance of resistance. Of the 12 instances of occurrence of the 4th most common allele in the set of 186 varieties, 11 were resistant varieties in the Holdfast/Moulin/Claire lineage. However, although our genetic mapping shows that Cadenza possesses *Sbm1* resistanceThe 12th occurrence was in a susceptible variety, Compal. None of the other three markers were correlated with resistance in the Holdfast-derived lineage or in any other.

Figure 7: Pedigree relationships and graphical genotypes of UK and European SBCMV-resistant lineages for a 10cM interval of chromosome 5DL sapping *Sbm1*. The graphical genotypes represent allelic status of four linked markers in consecutive coloured boxes. Yellow is used to indicate possession by the variety named above of the most common allele, green – the second most frequent allele, salmon – the third, and in the case of wmc765, we highlight the fourth commonest allele in red.



Discussion

The culmination of this project has been the identification by QTL mapping of two major genes of additive effect. A number of groups studying resistance to SBCMV or the closely related SBWMV are converging on a major resistance on chromosome 5DL. Bass *et al.* 2006 report the location of *Sbm1* from the Cadenza background, Perovic *et al*. 2005 are mapping in the Tremie background, and since Claire and Tremie are related cultivars, we assume the single gene detected in Tremie to be *Sbm1* (Perovic *et al*. 2005) a hypothesis strengthened by our chromosomal haplotype analysis. Narasimhamoorthy *et al*. 2006 have also identified a major QTL for resistance to the US SBWMV-*Q.Sbv.ksu-5D* - located on the distal end of 5DL in cultivar Karl92, and in independent work carried out in the US has revealed resistance – designated *Sbwmv1* - in the same region in the synthetic-derived background KS96WGRC40 (Hall 2006). We confirm here that the *Sbm1* locus is present in Cadenza as well as in Moulin/Claire-related UK varieties. However, the major surprise given previous reports of Bass *et al* (2006) was that a second major gene is also present in the Cadenza background and that it is expressed in the field and in our CE test, but not using the previously described CE test of Kanyuka *et al*, 2004.

The second gene we have identified and mapped, *Sbm2*, appears to confer equally effective, but more environmentally influenced resistance. Yet, when present in the same genotype with *Sbm1*, virus levels in leaves are significantly lower than when either gene is present alone (Figure 6b). We speculate that *Sbm2* therefore represents a block on viral replication or transmission/transport which is overcome in conditions defined by Kanyuka *et al* 2004. Our ability to reveal both genes in a single segregating population has revealed an additive interaction pointing towards the potential advantages This case underlines the strength of basing methodological development on sound field-based experimentation.

Although we are the first to have mapped *Sbm2*, some evidence for the action of two major genes has previously been observed. Barbosa *et al*. 2001 described the segregation of resistance to SBWMV in Brazilian cultivar Empraba16 as due to “the presence of two genes controlling the resistance reaction to WSBMV with a dominant epistatic effect. The result indicated a major dominant gene conditioning resistance and second dominant gene with a minor effect conditioning an intermediate reaction that was highly influenced by environment”.

The other major conclusion is that resistance is relatively commonly encountered in UK adapted wheats and together with the markers and phenotypic screening methods developed in this project, we have tools and resources and indications of abundant sources of resistance adapted hexaploid germplasm needed to protect wheat yields from potential spread of SBCMV throughout the UK.

DEVELOMENT OF A HYDROPONICS METHOD FOR SCREENING CULTIVARS FOR RESISTANCE TO SBCMV

Introduction

Screening wheat lines for field resistance to SBCMV is a lengthy process requiring a full 8-month season. Contained automatic immersion systems (AISs) have been used previously to successfully study the lifecycle and virus-vector interactions of SBCMV-related viruses (Dahn & Buchenauer, 1993; Legreve *et al*. 1998). At the outset of the project CSL had already developed a recirculating watering system to produce SBCMV infected plants to investigate the virus host range. The main objective of this study was to further develop and validate a method based on AISs for the rapid screening of SBCMV resistance in winter wheat lines. The validated method should mimic results obtained using conventional field studies.

Materials and Methods

Provisional optimisation of controlled environment (CE) phenotyping test

AISs were used to facilitate the rapid build-up of viruliferous *P. graminis* in the roots of test winter wheat lines, and therefore shorten SBCMV infection times. Each AIS unit was set-up using a similar system to that described by Legreve *et al*. 1998. An electric pond pump was attached to an electronic timer and used to re-circulate water from the lower reservoir tank to flood irrigate modules containing test lines planted in SBCMV-infected soil and suspended in the upper culture tank. To increase throughput, 70 ml module trays were placed onto 315 nm gauge nylon mesh, allowing 100 lines to be tested per AIS unit. Soil was collected from a field containing a natural inoculum source of SBCMV in Wiltshire, UK. The soil was air-dried and passed though a 2 mm sieve prior to mixing 70:30 with sterile sand. The soil:sand mixture was then placed in the modules sited within the upper culture tank of the AIS.

Two cultivars of winter wheat representing lines previously shown to be either resistant (Hereward) or susceptible (Deben) in field tests (Budge *et al*, 2002) were used to investigate the effect of different storage temperatures and test durations on virus titre in leaf and root material. All seed were pre-germinated in sterile distilled water for 24 hours prior to sowing 10 plants per cultivar into each of 8 replicate modules. For each experiment, a single module of each cultivar was harvested weekly from 5 to 12 weeks post planting. For each experiment the AIS units were placed in a controlled environment room (Sanyo-Gallenkamp) set at 80% relative humidity with an eight-hour photoperiod and a dawn and dusk function. The experiment was repeated five times using the following different day/night time temperature settings: 12°C/6°C; 12°C/12°C; 15°C/15°C; 18°C/18°C; 21°C/21°C. A provisional study showed altering the length of the irrigation period from 0.5 to 4.0 hours in every 24 hours had no effect on *P. graminis* infection (data not shown). The pump was set to flood irrigate the

upper culture tank for 2 hours in every 12 and the water in the lower reservoir tank was topped up weekly.

At harvest, root and leaf material were cleaned of any contaminating soil and frozen at –80°C until required. At the end of each experiment plant material was thawed and tested for the presence of SBCMV using triple antibody sandwich (TAS) ELISA as described in Ratti *et al*. 2004. Absorbance was measured at 405 nm wavelength (A_{405}) on a ThermoMax Microplate reader (Molecular Devices, USA) 2 hours after the addition of the substrate. Prior to the start of the experiment, known SBCMV positive material was homogenised, aliquotted into 0.5 g sub-samples and frozen at –80°C. A single aliquot of positive material was tested in quadruplicate wells for each 96 well microtitre plate.

Screening of winter wheat cultivars with known field reactions to SBCMV

Eighteen UK cultivars of winter wheat were tested using the optimised CE phenotyping test. Cultivars previously shown to have field resistance (Aardvark, Cockpit, Hereward, Phlebas, Xi 19) or susceptibility (Option, Richmond, Tanker) to SBCMV were included (Budge *et al* 2002; Bayles and Napier 2001). In addition, cultivars with unknown field reactions were tested (Dickinson, Einstein, Gladiator, Istabraq, Nijinsky, Robigus, Smuggler, Solstice, Welford, Wizard). For each cultivar two replicate modules were planted each with 10 plants. Eight replicate plots of a susceptible (Avalon) and a resistant (Claire) control variety were planted into the AIS unit.

In order to allocate a phenotype to each cultivar, the upper and lower 95% confidence intervals were calculated for respective resistant (Claire) and susceptible (Avalon) parents assuming the t-distribution. Cultivars with an A_{405} lower than the upper 95% confidence interval for the resistant parent were classed as resistant. Cultivars with an A_{405} greater than the lower 95% confidence interval for the susceptible parent were classed as susceptible. Cultivars with an A_{405} in-between the two values remained unclassified. Cultivars were scored according the consensus results from the replicate tests. For cultivars where one replicate remained unclassified, the single classified result (resistant or susceptible) was taken as the final phenotype

The field resistance reaction was assessed for each of the 18 UK cultivars using replicate tussock plots planted in a field situated on the farm in Wiltshire where first UK outbreak of SBCMV was confirmed in 1999 and where the trial plot area was known from previous work to be uniformly infected. Visual symptoms were assessed on a 0-4 scale where (0-4, where 0 = no symptoms and 4 = severe mottling and stunting). In addition, leaf samples were collected from each plot and tested for SBCMV using TAS ELISA as previously described. Leaf material was considered positive for SBCMV if the A_{405} exceeded three times the negative control. Symptom severity and ELISA data were pooled to give an

overall phenotype for the field test. Cultivars were classed as resistant if they showed no leaf symptoms coupled with an A_{405} less than three times the negative control. Cultivars were classed as susceptible if they showed strong leaf symptoms coupled with an A_{405} greater than three times the negative control. Cultivars where replicate field plots gave contrasting results remained unclassified.

Natural Field Infection Phenotyping

The AxC and CxM populations were phenotyped for resistance to SBCMV in the field in 2003/2004 and in 2004/2005. The XxS population was tested in the field in 2004/2005 only. Small plots, approximately 25 cm diameter, were hand-sown in the infected trial site. Either one (2003) or two (2004) replicate plots of each line, including both parents, were sown in October of each year. The SBCMV susceptible cultivar Consort was planted at intervals within each trial to confirm the presence of infective SBCMV within the trial area.

Visual symptoms of SBCMV infection were assessed in April and May of each season at GS30 and GS37-39 respectively. Disease incidence (% infected tillers) and disease severity (0-4, where 0 = no symptoms and 4 = severe mottling and stunting) were assessed at each visual inspection. At the second assessment plant material was sampled and tested for the presence of SBCMV using TAS ELISA as previously described. In 2004, two or three plants were harvested from each plot with roots intact and both root and leaf material tested separately for the presence of SBCMV using TAS ELISA. Viral titres in roots appeared not to correlate with visual symptom expression in the analysis of the 2003/2004 data due to difficulties in sampling roots in clay soil, therefore in 2004/2005 five random leaf samples were taken from each plot, bulked and tested using ELISA. In the 2005/2006 field season, a number of wheat cultivars representing members of the extended pedigree of known resistant cultivars were sown in replicated plots at the Wiltshire infected site and visual scores obtained as described above.

Phenotyping double haploid populations using the optimised controlled environment test

Three double haploid (DH) populations were screened using the optimised CE test. The first was a publicly available DH population, derived from a cross between Avalon (susceptible) and Cadenza (resistant), consisting of 203 lines. The second was a DH population consisting of 198 individuals from a cross between Claire (resistant) and Malacca (susceptible) supplied by Nickerson-Advanta UK Ltd. The third population consisted of 113 DH lines derived from a cross between Xi19 (resistant) and Solstice (susceptible) supplied by Nickerson-Advanta UK Ltd. At least 14 replicates of each parental line were included in each experiment to act as controls. Experiments on DH populations AxC and XxS were repeated twice and lines from CxM were repeated three times.

Statistical analysis of phenotypic data

Mixture models with estimated mixing proportions were fitted and then mixture models with mixing proportions constrained to 0.5 were fitted to the phenotypic segregation data. The fit of different mixture models was then compared with a likelihood rotation test (LRT). This is equivalent to chi-squared goodness of fit for cases where varieties can be classified as resistant or susceptible without error. Chi-squared goodness of fit to standard Mendelian ratios was tested for the Mendelized consensus phenotypic classification of each line considering all phenotypic evaluations carried out.

Results

Provisional optimisation of CE phenotyping test

The average of the duplicate A_{405} readings for each sample was expressed as a percentage of the average of the four positive control wells from the plate within which the samples were tested. Average A_{405} readings for positive controls ranged from 1.38-3.27. The majority of leaf tissue from both cultivars contained consistently low levels of SBCMV with values ranging from 6.0-10.5% and 5.9-12.3% of the positive control for respective resistant cultivar Hereward and susceptible cultivar Deben. Sporadic high virus titres were recorded in leaf material from cultivar Deben for week 11 at 12°C/12° and week 9 at 15°C/15° with respective values of 95.5% and 171.4% of the positive control.

In the experiments conducted at 12°C/6° and 12°C/12° the majority of root tissue from resistant cultivar Hereward contained consistently lower levels of SBCMV than was recorded in the root material of the susceptible cultivar Deben. The level of SBCMV in Deben roots was greatly reduced in experiments conducted at 18°C/18° and 21°C/21°C, where the level of SBCMV was not notably higher than that found in Hereward (Figure 1). Larger more consistent differences were found between Deben and Hereward using root material than leaf material (Data not shown). In addition, temperatures of 15°C and higher reduced the root titre in the susceptible cultivar Deben. Finally there was no obvious advantage in running the test for longer than 8 weeks.

The optimised CE test protocol used for the remainder of the project was a consistent day and night temperature of 12°C for eight weeks, after which the root material was tested for the level of SBCMV using TAS ELISA.

Screening of winter wheat cultivars with known field reactions to SBCMV

All cultivars previously reported as resistant or susceptible to SBCMV in field studies were classified correctly using the CE phenotyping test. All ten cultivars with unknown field reactions proved to be susceptible in both field and CE phenotyping. Cockpit had contrasting results in each replicate field

plot and remained unclassified using the field data in this study (Table 1). Clear resistant and susceptible clusters were identified when mean A_{405} values from field and CE tests were plotted (Figure 2).

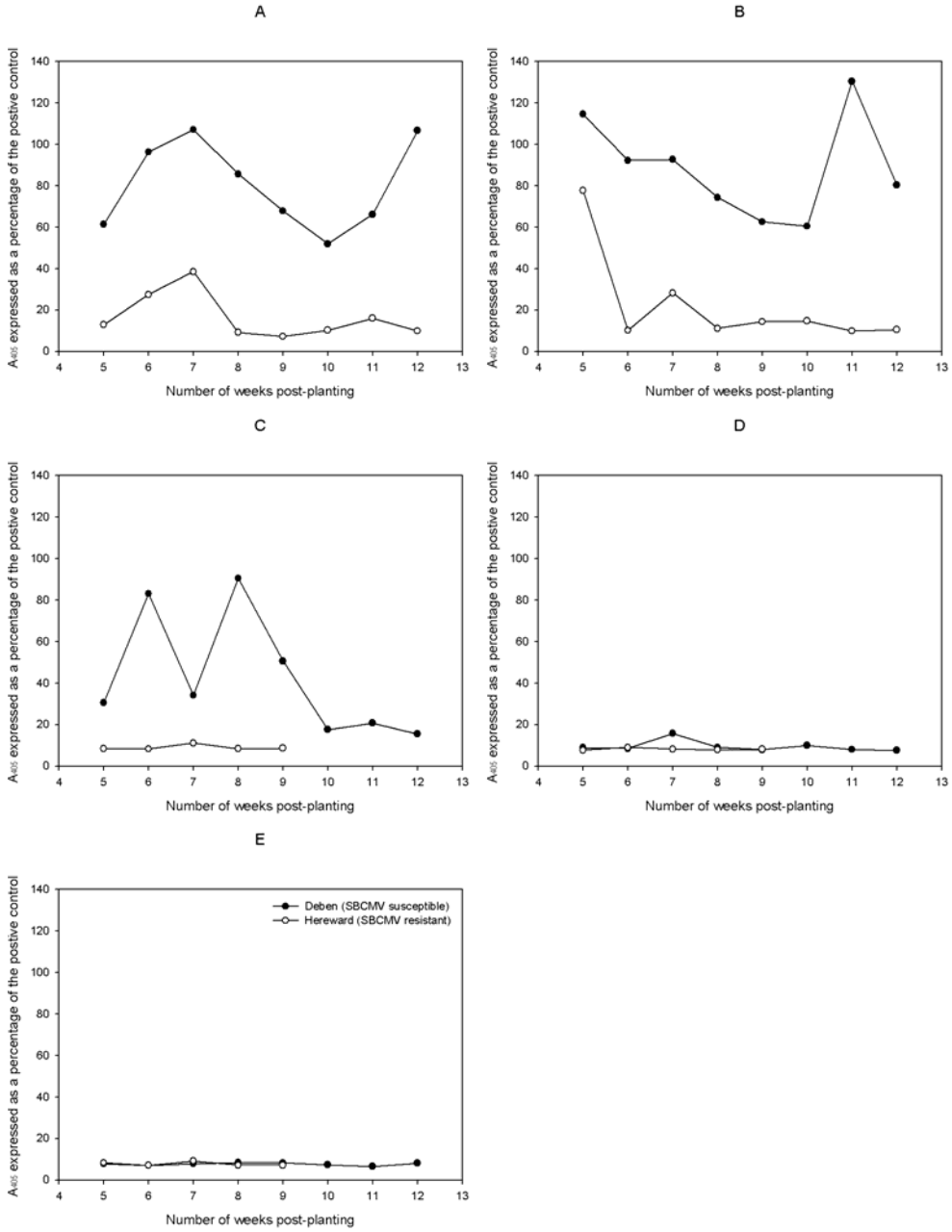


Figure 1. Results from the provisional optimisation study using AISs within a controlled environment cabinet set at 80% relative humidity with an eight-hour photoperiod and a dawn and dusk function, to investigate the effect of temperature and time on the level of SBCMV in root tissue from cultivars Deben (susceptible) and Hereward (resistant). A_{405} values from the SBCMV specific TAS ELISA are presented as a percentage of the positive control. Each data point represents the result of a single module containing 10 plants. Results are presented from five experiments conducted at different day/night time temperature settings: (A)12°C/6°C; (B) 12°C/12°C; (C) 15°C/15°C; (D) 18°C/18°C; (E) 21°C/21°C. Data were missing for cultivar Hereward for weeks 10-12 in experiments C-E.

Table 1 Comparison of absorbance values (A_{405}) from TAS ELISA tests for SBCMV using leaves collected from two replicate field plots with values from root material from replicate experiments using AISs in a controlled environment (CE). Where available, information on previously reported field reactions is included; (R) resistant; (S) susceptible; (N) not previously tested. Absorbance values for negative wheat ranged from 0.069-0.010. Replicates from field plots were consistently R or S except for Cockpit, which exhibited a mixed reaction and therefore remained unclassified in this study (U). For the CE test, resistant and susceptible controls consisted of eight replicate plots and 95% confidence intervals were calculated assuming the t-distribution. Cultivars were assigned as resistant (R) or susceptible (S) phenotype based on the consensus result from four replicate tests; for cultivars where one replicate remained unclassified, the single classified result (resistant or susceptible) was taken as the final phenotype.

Cultivar	**A_{405} from leaf material collected from field plots**		**SBCMV resistance phenotype**	**A_{405} from root material grown in AISs and associated CE test result**		**SBCMV resistance phenotype**
	Rep. 1	**Rep. 2**	**from field test**	**Rep. 1**	**Rep. 2**	**from CE test**
Aardvark ($R^{1,2}$)	0.088	0.102	R	0.098	0.089	R
Cockpit ($R^{1,2}$)	0.082	1.160	U	0.091	0.260	R
Hereward ($R^{1,2}$)	0.081	0.087	R	0.168	0.248	R
Phlebas (R^{2})	0.095	0.078	R	0.106	0.200	R
Xi 19 ($R^{1,2}$)	0.107	0.083	R	0.238	0.102	R
Option ($S^{1,2}$)	0.892	0.894	S	0.380	1.366	S
Richmond (S^{2})	0.668	0.663	S	1.028	0.804	S
Tanker ($S^{1,2}$)	1.883	0.814	S	1.526	0.572	S
Dickson (N)	0.611	0.476	S	0.550	0.620	S
Einstein (N)	0.767	1.058	S	0.415	0.805	S
Gladiator (N)	1.567	1.048	S	0.623	1.403	S
Istabraq (N)	1.016	1.186	S	1.487	1.086	S
Nijinsky (N)	0.669	0.806	S	1.197	0.501	S
Robigus (N)	0.680	0.455	S	0.971	0.830	S
Smuggler (N)	0.866	0.624	S	0.362	0.917	S
Solstice (N)	0.658	0.755	S	0.813	0.886	S
Welford (N)	0.943	0.972	S	1.049	0.329	S
Wizard (N)	1.000	0.464	S	0.637	2.142	S
Resistant control	-	-		0.245(0.172,0.319)		-
Susceptible control	-	-		0.739(0.490,0.988)		-

Phenotype based on data from previous studies [1]Budge *et al* 2002; [2]Bayles and Napier 2001

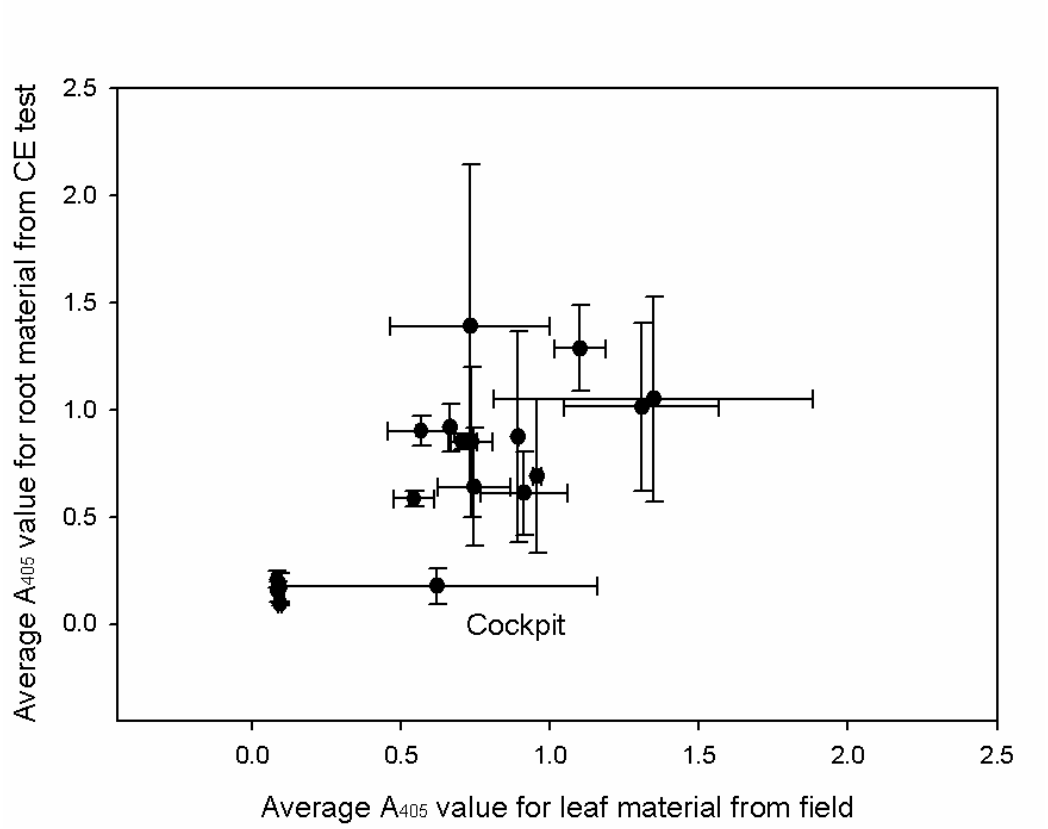


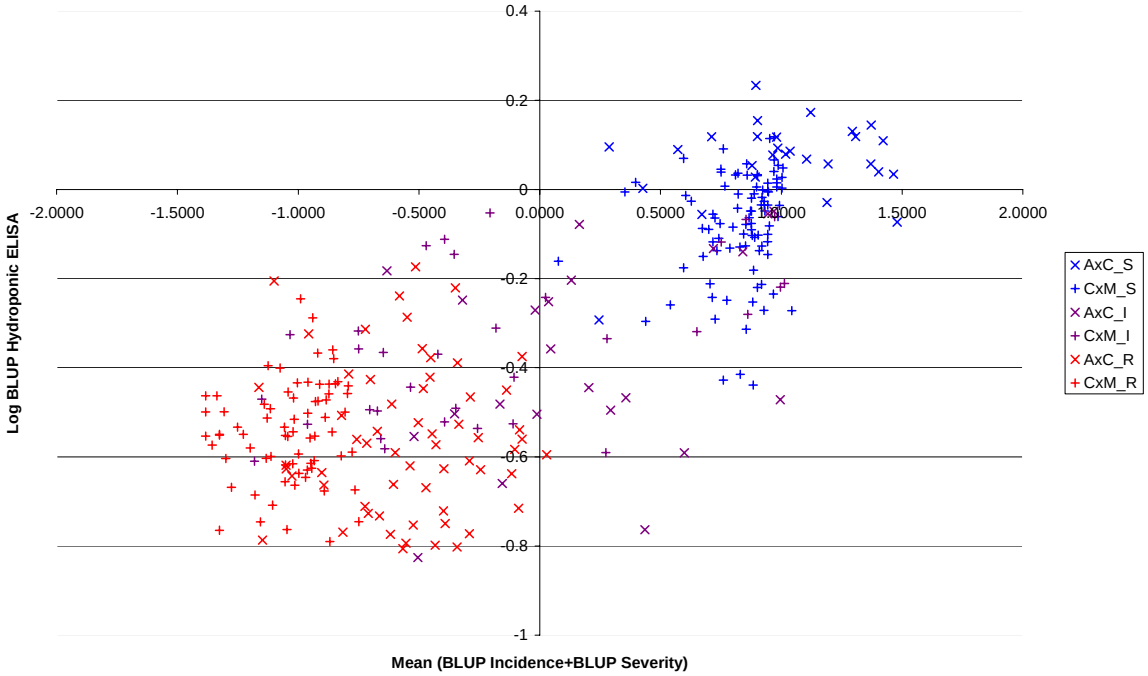
Figure 2: Average absorbance values (A_{405} with corresponding standard errors from TAS ELISA tests for SBCMV for 18 cultivars of winter wheat. A_{405} values are presented from testing duplicate samples of root material using the CE method and from leaf material collected from duplicate plots grown in a field containing a natural inoculum source of SBCMV. Clear clusters of SBCMV resistant (bottom left) and susceptible (top right) cultivars are formed. The results for Cockpit (recognised previously as a resistant cultivar) gave a mixed reaction in the field with alternate replicates showing a resistant and a susceptible reaction.

Correlation of field and controlled environment (CE) evaluation of resistance in two segregating populations

In order to study the genetic control of previously observed field resistance to SBCMV in Claire and Cadenza winter wheat cultivars, we separately obtained replicated measures of SBCMV reaction of lines from AxC and CxM segregating populations using three different methods: 1) visual scoring of symptoms in field trials in 2004 and 2005, 2) ELISA on leaf tissue collected from visually scored field plots, and 3) ELISA on leaf tissue collected from material artificially inoculated in the AIS described above. The different measures of SBCMV reaction were subjected for the first time to a rigorous correlation in immortalised populations segregating for field-based resistance from two distinct sources of resistance. Incidence and Severity visual scores were very highly correlated (R^2 = 0.9188), as too were ELISA values from either field or hydroponic tests (R^2 = 0.837). The lowest R^2

was obtained, not surprisingly, from correlation of hydroponic ELISA values and aggregate visual symptom score. The latter correlation is represented in the scattergram in Figure 3 with datapoints from the respective segregating populations separately identified. Across the combined populations and a total of 307 lines, the overall R^2 was 0.648, with the CxM RILs population showing a better correlation (R^2 = 0.744) than AxC DH population (R^2 = 0.599).

Figure 3: Correlation between virus levels determined separately in the controlled environment test and in the field for 307 lines of the combined AxC and CxM populations. Datapoints relating to lines whose consensus score was resistant (R), susceptible (S), or intermediate (I) are separately identified. BLUP = Basic Linear Unbiased Predictor



Inheritance of SBCMV resistance and segregation ratios in three segregating populations

Heritabilities were high in each population (Table 2) ranging from 0.757 (AxC field) to 0.874 for CxM (log All), from which we conclude that the phenotype, although influenced by environmental factors, should be governed to a large extent by genetic factors. Thus we looked at the feasibility of mapping the resistance as a major gene or genes.

Two methods were used to assess the likely number of genes underlying the resistance phenotype. Firstly, when each individual line was classified phenotypically as either resistant, susceptible or unknown based on the consensus phenotype when all measures of SBCMV were considered together, chi-squared analysis showed that both the CxM and XxS mapping populations exhibited R:S ratios

consistent with 1:1 segregation indicative of single Mendelian genes, while the AxC population (with Cadenza as resistance donor) showed a R:S ratio consistent with either 2:1 or 3:1 segregation suggesting the presence of more than one independent genes or QTL of major effect (data not shown). Secondly, in order to avoid the subjectivity in Mendelization of phenotype, mixture models were fitted to the data. CxM was not significantly different from a 1:1 ratio indicating segregation of a single major gene. XxS was not significantly different from a 3:1 ratio indicating two interacting QTL, while AxC data fits poorly to both a 1:1 ratio for two phenotypic groups, and to a 2:1:1 ratio for three phenotypic groups. 100% of CxM lines and 93% of AxC lines can be confidently ($p<0.05$) classified in this manner as either resistant or susceptible using these mixture models. We conclude from this first level of phenotypic analysis that a) despite the evident differences in exposure of samples to environmental variation, the CE and field-based phenotypic measures are highly correlated and b) there are both major and minor gene effects on SBCMV reaction in these populations, as evidenced by the obvious bimodal distribution overlaid with a continuum of the phenotypic values consistent across different measures.

Table 2: Heritabilities for CxM and AxC segregating populations

Population	Phenotypic measure	mean h^2
AxC	field	0.757
AxC	log field	0.811
AxC	hydro	0.855
AxC	log hydro	0.837
CxM	field	0.792
CxM	log field	0.830
CxM	All	0.853
CxM	log All	0.874

Since all three populations were in the fully homozygous state, we cannot comment on the dominance or recessivity of the resistance alleles, and experiments are in progress to clarify this point.

Discussion

The continued management of SBCMV requires the availability of high yielding wheat cultivars with field resistance to the virus. The traditional approach to screening wheat cultivars for resistance to SBCMV is grown on virus infected land for an entire season, assessing the plants for both symptom development and virus titre in leaf material. Field-testing is time consuming and limits the number of cultivars which can be tested. A CE test was developed for the rapid screening of winter wheat lines for resistance to SBCMV. The CE test correctly classified the resistance phenotype for 5 resistant and 13 susceptible cultivars of UK winter wheat in only 8 weeks. The CE method offers a novel approach because root material is tested rather than leaf material. More rapid build-up of SBCMV in the roots of susceptible compared to field-resistant cultivars was first observed in France in 1984. Root exudates from cultivars demonstrating field resistance had far lower ELISA absorbance values than cultivars showing severe symptoms in the field when samples were taken early the following January after an autumn planting (Hariri *et al*, 1987; Lapierre *et al*, 1985). Quantitative molecular testing of root material from plants grown in Wiltshire suggested significantly lower titres of SBCMV in root material of resistant when compared to susceptible cultivars (Ratti *et al*. 2004). Such data suggests the resistance mechanisms of SBCMV acting in UK winter wheat are more complex than simply reducing virus accumulation in foliar tissues.

The absence of SBCMV in root and leaf material from both resistant and susceptible cultivars at and above 18°C was recorded in the initial validation experiment. This observation suggests the increased temperature has disrupted the infection cycle of SBCMV and could explain why symptoms of SBCMV tend to fade as temperatures rise in the summer months. Possible points of disruption could be the failure of *P. graminis* to infect the roots or less efficient virus replication and movement.

An alternative method for the rapid screening for resistance to SBCMV has been proposed involving the testing of leaf regrowth after initially removing all aerial plant parts (Kanyuka *et al*. 2004). Using this method, Kanyuka *et al*. have proposed a 1:1 segregation ratio of resistant to susceptible lines for the same AxC derived DH population used in the current study (Kanyuka *et al*. 2004). The authors went on to map this gene, designated *Sbm1* to chromosome 5DL (Bass *et al*. 2006). Interestingly, for the same population, our data, obtained both by observation of symptoms expressed in field conditions and in a carefully developed CE test, consistently detect a higher number of resistant plants than Kanyuka *et al.* 2004. Therefore, a line-by-line comparison of the data for individuals in their study was made using the raw data included in that publication. Whereas resistant lines from Kanyuka *et al*. 2004 are all (except for one line) resistant in our population screening, the Kanyuka *et al*. 2004 susceptible lines segregate 1:1 for resistance in our tests (approximately half of the Kanyuka *et al*. 2004 susceptible lines are resistant in our field and hydroponic screening). This suggests that one

common resistance gene (*Sbm1*) is detected by both approaches, while an additional unlinked resistance locus is detected in the field. Furthermore, it appears that the hydroponic inoculation system we have developed is a closer match of the field conditions than previously developed protocols demonstrating the importance of including comparative data from field trials to validate any potential screening method based in the laboratory.

The XxS (with Xi19 as donor of resistance) population is likely to segregate for at least one of the Cadenza resistance genes as Cadenza is a parent of Xi19.

INVESTIGATION OF THE MECHANISM OF RESISTANCE TO SBCMV IN UK CULTIVARS USING REPRESENTATIONAL DIFFERENCE ANALYSIS

Introduction

Representational Difference Analysis (RDA) uses the technique of subtractive hybridisation to allow direct comparison of two populations of mRNA and enriches sequences that more highly expressed in one population than in the other. The process involves two successive hybridisations of cDNA from the two populations; sequences that are not hybridised and therefore more highly expressed in the tester population, are enriched and cloned for later analysis. The main advantages of RDA are that no prior sequence information is required and that RDA can identify even rarely expressed sequences, which are often missed by other methods. For RDA to be successful it is important that any variation external to the experiment is kept to a minimum, therefore identified sequences are more likely to be expressed as a direct result of the experimental conditions. To achieve this two varieties of wheat were chosen that are very closely related however, one "Claire" is resistant to SBCMV and the other "Istabraq" is susceptible to SBCMV. In this study the expression of both varieties was compared under infection with SBCMV to try and isolate sequences involved in the resistance to SBCMV.

Materials and Methods

Representational difference analysis

A provisional time-course experiment suggested that SBCMV was present in a higher titre in Istabraq roots than in Claire roots after 12 days, indicating the resistance mechanisms were activated at this early stage. CTAB total RNA extractions were prepared from root samples taken 12 days after infection with SBCMV for cultivars Claire and Istabraq, these RNA extractions were pooled and the mRNA purified using Qiagen's Oligotex mRNA purification kit. RDA was carried out in the forward direction using Claire (the resistant variety) as the tester and Istabraq (the susceptible variety) as the driver, in this way sequences more highly expressed in Claire than in Istabraq were selected. The RDA was also carried out in the reverse direction with Istabraq as the tester and Claire as the driver to select sequences more highly expressed in Istabraq. This technique allows for the detection of sequences both up-regulated (forward direction) or down-regulated (reverse direction) in the resistant variety Claire. RDA was carried out using Clontech's PCR-select cDNA subtraction Kit available from BD biosciences. The steps performed in this procedure briefly comprised: first strand and second strand synthesis; Rsa I restriction endonuclease digestion to create short blunt ended molecules of dsDNA; ligation of adaptors; two rounds of hybridisation and finally two rounds of PCR to amplify differentially expressed sequences. The resulting DNA fragments were cloned into Promega's pGEM T vector.

Screening of potential difference product.

Initial screening of potential difference products was carried out using Clontech's PCR- Select Differential Screening Kit. Traditionally clones were randomly selected and used in northern blot analysis. This kit allowed many clones to be screened simultaneously for their differential expression by dot blot hybridisation. The use of [α-^{32}P]dCTP was recommended to produce radiolabelled probes, however for safety reasons this was replaced with non-labelled dCTP, the probes produced were then labelled with Amersham Bioscience's Alkphos Direct Labelling and Detection system, which utilises a chemiluminescent rather than radioactive detection system. Initially 96 forward subtracted and 61 reverse subtracted clones containing potential difference products were screened. The inserts from these clones were amplified and spotted onto four membranes before being probed with four separate chemiluminescent probes. Differentially expressed clones produced from the forward subtracted library should only hybridise to probes derived from Claire and clones from the reverse subtracted library should only hybridise to Istabraq derived probes. Two of the probes were produced from the same Claire and Istabraq cDNA populations that were used in RDA. This approach is widely used but can often miss less abundant sequences. Therefore to detect less abundantly expressed sequences, a further two probes were produced from the whole difference forward and reverse subtracted libraries themselves. However, there is an increased risk of false positives with the latter method.

Due to plasmid recombination events thought to have taken place in the clones during *E. coli* growth phases, cDNA libraries produced from RDA were retransformed into DH5-1α competent cells (Invitrogen), which are more resistant to recombination events. In addition the *E. coli* were grown in the presence of 1% glucose to completely switch off the lac promoter and prevent the translation of any of the insert sequences which could be harmful to the cell and lead to recombination of the plasmid. In this second round of screening 56 clones from the forward subtracted and 16 clones from the reverse subtracted libraries were screened. Of these clones, 7 were selected and their inserts screened; 6 clones were from the forward subtracted library and 1 from the reverse subtracted library.

Analysis of potential differentially expressed sequences

Clones identified as containing differentially expressed sequences by dot blotting were sequenced. Any plasmids which were found to have recombined during the *E. coli* growth phases had vector sequences removed using VecScreen (NCBI) to produce up to 5 separate none vector sequences for each clone. The sequences obtained were used to search for any similarity with known sequences on public access databases: BLASTn[b], TIGR[a], BLASTx; possible proteins predicted using Genescan and predicted peptides used to search BLASTp.

[b] http://www.ncbi.nlm.nih.gov/BLAST/

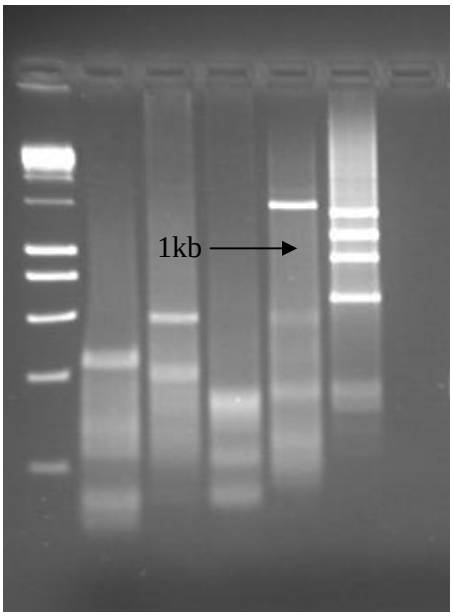
Analysis of potential difference products by real-time RT PCR

Several difference products were cloned multiple times, suggesting the Claire cDNA population contained a high concentration of these difference products. Two such products were selected for more detailed investigation C sub A9 (3 clones) and C sub G1 (4 clones). Assays for use with real-time RT PCR (using TaqMan® chemistry) were designed to the two sequences. A time-course experiment was set up whereby modules of Claire and Istabraq (5 plants in each module) were planted in soil either infected with SBCMV or in uninfected soil within a AIS as described in the CE phenotyping test. In addition 5 resistant and 5 susceptible isogenic lines from the Avalon x Cadenza population were planted in only infected soil (due to a small amount of available seed). Triplicate samples were taken at 5, 7, 9, 12, and 14 days after planting and tested both with a SBCMV specific real-time RT PCR assay and with the C sub A9 and C sub G1 assays. An RNA specific assay designed to the protein disulphide isomerase gene (PDI) gene was used as an RNA specific internal control and for normalisation of the results.

Results

Representational difference analysis

DNA fragments obtained by RDA (Figure 8) were cloned into pGEM T easy vector and initially 96 forward and 61 reverse subtracted clones were randomly selected and their inserts amplified (Figure 9).



[a] http://tigrblast.tigr.org/euk-blast/index.cgi?project=tae1

Figure 8: DNA fragments produced after representational difference analysis lane 1 marker, lane 2 Forward subtracted, lane 3 unsubtracted Claire control, Lane 4 reverse subtracted, lane 5 unsubtracted Istabraq control, lane 6 positive control, lane 7 negative control.

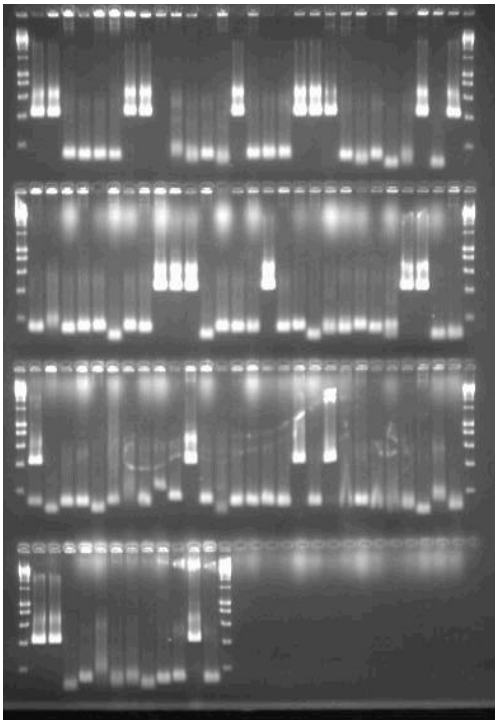


Figure 9: Amplified inserts from forward subtracted clones.

Screening of potential difference products

After initial screening by dot blot hybridisation, 15 forward subtracted and 6 reverse subtracted clones were selected and their inserts sequenced. Analysis of these sequences revealed that recombination events had taken place in 10 of the clones and the inserts contained sections of vector sequence. The vector sequence was identified and removed and the fragments of insert DNA sequence were saved separately producing a total of 40 sequences from the first round of screening. A second round of screening from randomly selected clones produced a further 7 sequences, none of which had undergone recombination events, 6 from the forward subtracted and 1 from the reverse subtracted library.

Analysis of potentially differentially expressed sequences

Full details of all sequences and any similarities to sequences published on public access databases are shown in Appendix 1. Of the 47 clones sequenced only 6 produced no similarity with any of the databases searched. Four clones (C sub F2c, C sub A1, C sub B3, and C sub G1) were found to contain the same insert sequence which had a similarity with a 42bp region of *Oryza sativa* genomic

DNA, chromosome 2, and similarity to wheat clone CA733072 on the TIGR database. Clone C sub A9 had similarity to a predicted mRNA-gibberellin responsive gene found in rice. Clone C sub A9 was found to be almost identical to 2 other sequences C2 sub G3 and C1 sub G12. Five sequences showed similarity with aquaporin and a further 4 sequences showed similarity with photosystem I P700 apoprotein A1 identified using the TIGR database. Other similarities identified included ATP synthase, hsp70, 14-3-3 protein, Mini-chromosome maintenance 7, Histone H3-like protein, and 18S rDNA. A further 8 sequences showed similarity to proteins or sequences with unknown function.

Analysis of potential difference products by real-time RT PCR

TaqMan® assays were designed to the sequences C sub A9 (similar to gibberellin responsive genes) and C sub G1 (analogous to a short section on chromosome 2 of *Oryza sativa*). Real-time RT PCR of Claire and Istabraq over a time-course after planting in SBCMV infected soil showed similar results to the pilot time-course study. Fourteen days after planting in SBCMV infected soil virus quantity in Istabraq roots rose to a higher level than found in Claire roots (Figure 10).

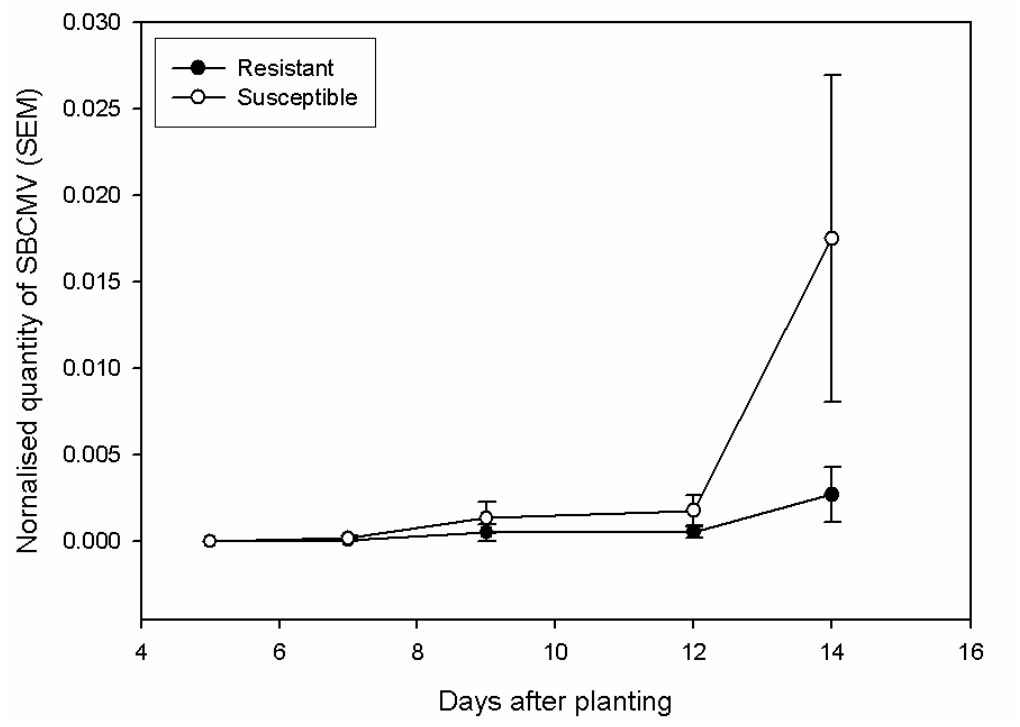
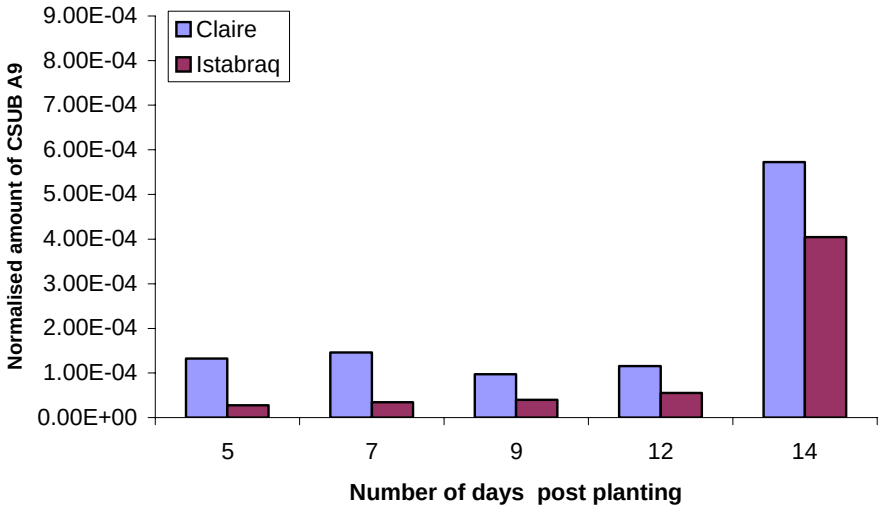


Figure 10: Normalised quantities of SBCMV in root tissue from 5 x resistant and 5 x susceptible isogenic lines from the Avalon x Cadenza double haploid population. For each line, triplicate modules were grown in AIS containing soil infected with SBCMV. Each module contained 5 individual plants. Root material was harvested after 5, 7, 9, 12 and 14 days and tested using real-time RT PCR. The

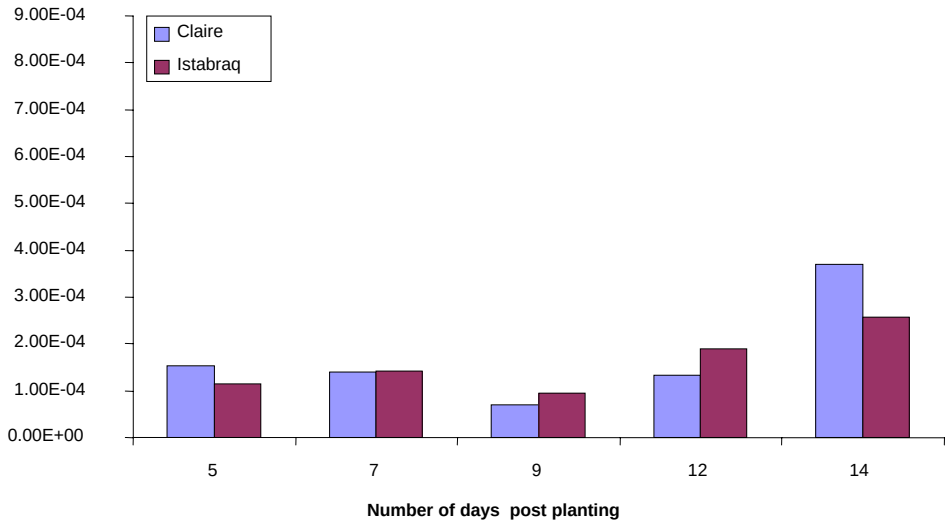
quantity of SBCMV was normalised using an RNA specific real-time PCR designed to the PDI gene of wheat.

Real-time RT PCR results for the insert from clone C sub A9 suggested an increase in expression at 14 days in all the lines tested, with consistently higher levels recorded in resistant lines. However, this pattern was repeated in both infected and uninfected soil, suggesting C sub A9 is not involved in any SBCMV resistance mechanism(s) (Figure 11).

(a)



(b)



(c)

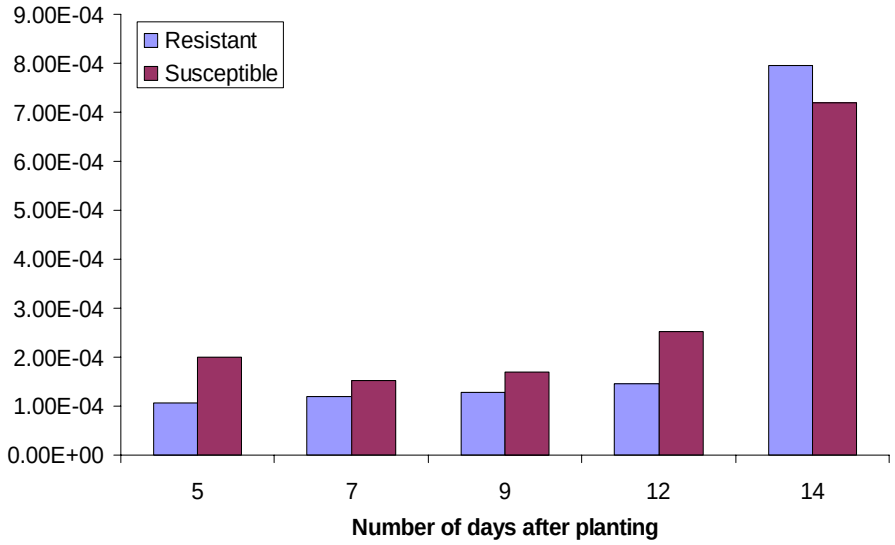
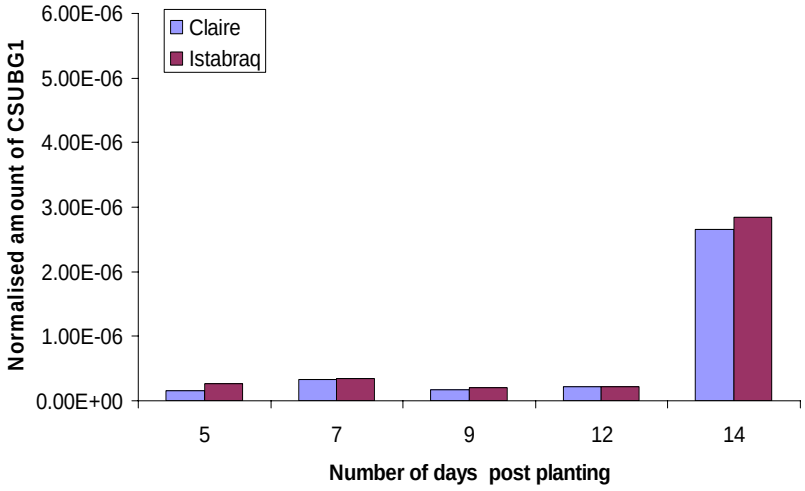


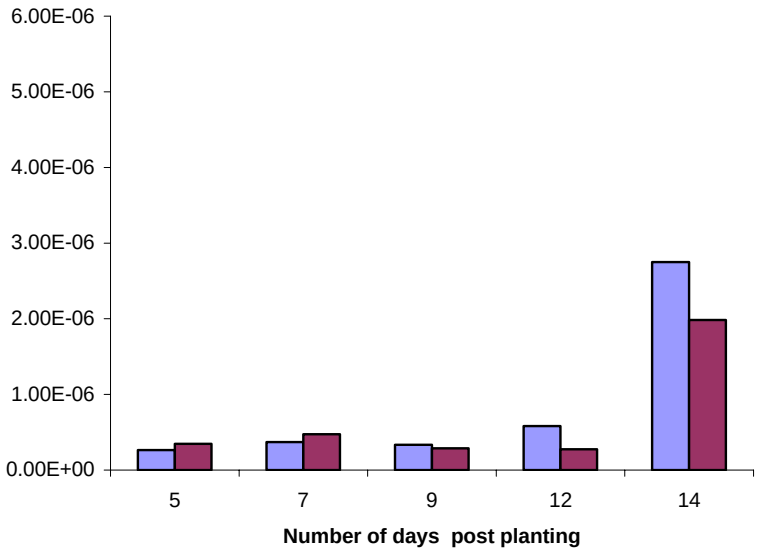
Figure 11: Normalised quantities of C sub A9 in root tissue from (a) triplicate modules of Claire and Istabraq planted in uninfected soil; (b) triplicate modules of Claire and Istabraq planted in SBCMV infected soil and (c) 5 x resistant and 5 x susceptible isogenic lines from the Avalon x Cadenza double haploid population planted in SBCMV infected soil. Modules were grown in an AIS and each module contained 5 individual plants. Root material was harvested after 5, 7, 9, 12 and 14 days and tested using real-time RT PCR. The quantity of C sub A9 was normalised using an RNA specific real-time PCR designed to the PDI gene of wheat.

Real-time RT PCR results for the insert from clone C sub G1 suggested an increase in expression at 14 days in all the lines tested. Interestingly, in the presence of SBCMV, the expression of C sub G1 was higher in resistant isogenic lines and Claire than the respective susceptible lines on test. This pattern was not repeated in uninfected soil suggesting C sub G1 is probably involved in the SBCMV resistance mechanism(s) (Figure 12).

(a)


6.00E-06
5.00E-06
4.00E-06
3.00E-06
2.00E-06
1.00E-06
0.00E+00
Claire
Istabraq
Normalised amount of CSUBG1
5
7
9
12
14
Number of days post planting

(b)


6.00E-06
5.00E-06
4.00E-06
3.00E-06
2.00E-06
1.00E-06
0.00E+00
5
7
9
12
14
Number of days post planting

(c)

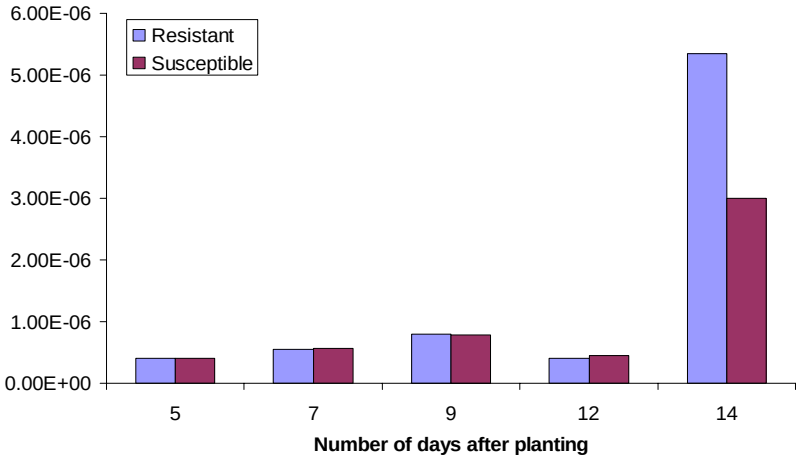


Figure 12: Normalised quantities of C sub G1 in root tissue from (a) triplicate modules of Claire and Istabraq planted in uninfected soil; (b) triplicate modules of Claire and Istabraq planted in SBCMV infected soil and (c) 5 x resistant and 5 x susceptible isogenic lines from the Avalon x Cadenza double haploid population planted in SBCMV infected soil. Modules were grown in an AIS and each module contained 5 individual plants. Root material was harvested after 5, 7, 9, 12 and 14 days and tested using real-time RT PCR. The quantity of C sub G1 was normalised using an RNA specific real-time PCR designed to the PDI gene of wheat.

Discussion

RDA has been used to identify sequences that are associated with resistance to SBCMV in resistant winter wheat variety Claire. A total of 47 DNA fragments were isolated by RDA and comprehensive analyses performed to identify similarities with published sequences or proteins. Two assays real-time RT PCR assays (TaqMan) were designed to sequences C sub A9 and C sub G1. C sub A9 was selected as it was found to be similar to gibberellin responsive proteins, this similarity was detected in 3 of the 47 sequences. The second sequence C sub G1 was found in 4 of the 47 clones and had similarity with both a region of the rice chromosome 2 sequence and with an unknown wheat clone. Twenty three of the initial 96 clones screened from the forward subtracted library contained an insert the same size as C sub G1 and it is assumed that this sequence may be much more highly expressed in the resistant variety and therefore important to investigate. Real-time RT PCR of the quantity of SBCMV over a time course of infection revealed that differences in the quantities found in resistant and susceptible lines start to be observed at 14 days and it is therefore assumed that day 14 may be a crucial day in the mechanisms of resistance. Investigation of C sub A9 using real-time RT PCR

revealed that although it was more highly expressed in resistant than in susceptible lines, and expression is increased in all varieties at day 14, the same pattern is also seen in uninfected plants. It is therefore believed that C sub A9 is unlikely to be involved in resistance mechanisms and may be involved in development of the plant. The expression pattern of C sub G1 however appears to be more interesting; little or no difference is observed between the varieties in uninfected soil, and again the quantities from both varieties is increased at day 14. Under SBCMV infection Claire shows higher expression levels at day 14 than the susceptible Istabraq, at the same timepoint with isogenic lines the difference between resistant and susceptible varieties is even greater. It is likely that this sequence may be associated to the resistance of some wheat varieties to SBCMV.

In addition to the highlighted C sub G1 sequences RDA produced many other sequences which could yield very interesting results. Many of the sequences identified were found in several of the clones for example similarity to the proteins aquaporin (major intrinsic protein (MIP) that forms pores in the membrane) and photosystem I P700 apoprotein A1. RDA also identified 8 sequences corresponding to unknown proteins and sequences identified in other studies. A further 6 sequences revealed no similarity with any known sequences and are novel products. A bioinformatics approach to in silico mapping of the wheat genes identified by RDA was undertaken in case any of the RDA products mapped to the Sbm1 region, thus making it a positional and functional candidate gene. However, only a few RDA sequences gave convincing matches to wheat TCs with highly significant reciprocal hits to rice orthologues, and no putative positional candidates were identified (data not shown).

A great deal of resources would be required to further investigate all of the sequences isolated by RDA, however this study has demonstrated that RDA is a powerful tool to identify genes more highly expressed in one mRNA population than in another, and has proved itself to be a useful method to identify sequences that may be involved in the mechanisms of resistance to SBCMV in wheat.

INVESTIGATION OF RESISTANCE OF UK CULTIVARS TO OTHER RELATED SOIL-BORNE VIRUSES

Introduction

The aim of this part of the study was to explore the possibility that resistance to SBCMV also confers resistance to other related soil-borne viruses. SBWMV is the type member of the *Furovirus* genus, and until recently was thought to be identical to SBCMV. However, recent molecular analysis (Diao *et al.*, 1999; Koenig and Huth, 2000ab; Yang *et al.*, 2001) has resulted in the International Committee on Taxonomy of Viruses approving a taxonomic proposal to divide the American and European isolates into different species within the *Furovirus* genus. New species have been denominated *Soil-borne wheat mosaic virus* (USA) and *Soil-borne cereal mosaic virus* (Europe). However, it is not known whether resistance to SBCMV will be effective against the closely related SBWMV.

Wheat spindle streak mosaic virus (WSSMV) is a *Bymovirus* in the family *Potyviridae*. Like SBCMV and SBWMV, WSSMV is also vectored by *P. graminis* and can cause serious disease on winter wheat. Although not yet detected in the UK, WSSMV is widely distributed across Europe and is often found infecting wheat in combination with SBCMV. Field trials on WSSMV land suggested UK cultivars could be resistant to this virus (Budge *et al*., 2002).

Aubian wheat mosaic virus (AWMV) is a serologically distinct virus with rod shaped particles found in France. The vector is not known, but *P. graminis* was found in the roots of the infected plants. A virus with identical serological properties was identified in a field in the UK in 1999 (Clover *et al*., 1999; Hariri *et al*., 2001).

Data have been published showing cultivar reactions to several soil-borne viruses (Armitage *et al*., 1990; Budge *et al*, 2002; Bayles and Napier, 2002; Hariri *et al*., 2001). We proposed to test cultivars from France, the UK and the USA for resistance to AWMV, SBCMV, SBWMV. Such a comparison should allow us to draw conclusions as to whether resistance genes that are suitable for one virus may confer resistance to other viruses.

Materials and Methods

Soil was collected from three fields, the first known to contain AWMV in France, the second SBCMV from the UK and the third a mixture of SBWMV with WSSMV collected from the USA. Each soil was diluted 50:50 with sterile sand (2EW) and placed in separate AISs as described previously in the CE phenotyping test. Thirteen cultivars of wheat with known resistance reactions to at least one virus were planted into the soil of each AIS (Table 4). Each AIS represented a separate experiment with four replicate modules of each cultivar set as a complete randomised block. Each module consisted of

10 replicate plants. The apparatus was left at 15°C within a controlled environment room for 14 weeks prior to root and leaf material being harvested and tested for the presence of the respective virus.

ELISA formats varied for the detection of each virus. A double antibody sandwich (DAS) ELISA was used for the detection of AWMV as described by Hariri *et al*. (2001). WSSMV was detected using a DAS ELISA protocol. Microtitre plates (Nunc maxisorp, 96 well) were coated with polyclonal anti-WSSMV (DSMZ, Germany) diluted 1:1000 in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$; pH 9.6) at 100 ml per well and incubated for 2 h at 33°C. Wells were washed with PBSt (20mM sodium phosphate, 150 mM sodium chloride pH 7.4 containing 0.05% Tween 20) on a plate washer (Skanwasher 300, Molecular Devices). Approximately 1 g of plant tissue was homogenised using a Lenze (Bioreba AG) grinder in 5 ml extraction buffer (PBSt, 0.1% Tween 20, 0.1% Marvel dried milk powder) and 100 ml added to duplicate wells on the coated plates. Plates were incubated at 4°C overnight and then washed as previously described. Alkaline phosphatase conjugated WSSMV polyclonal was then diluted 1:1000 in PBSt including 0.2% bovine serum albumin (BSA) (Sigma) and 100 ml was added per well. The plate was incubated at 33°C for 1 hour prior to washing as previously described. Substrate (P-nitrophenyl phosphate hexahydrate disodium salt (1 mg/ml)) was dissolved in substrate buffer (10% diethanolamine; 0.02% $MgCl_2$; pH 9.8) and 100 ml added per well. Plates were then incubated at room temperature for 2 hours and A_{405} was read on a Thermo Max microplate reader (Molecular Devices).

The detection of SBWMV was achieved by following the TAS ELISA protocol described in Ratti *et al* (2004). To enable the specific detection of SBWMV monoclonal antibody SCR132, which has been reported to only detect SBWMV, was substituted for monoclonal SCR133, which is know to detect both SBCMV and SBWMV (Chen *et al*., 1997). Duplicate samples of healthy roots were added to each 96-well micro titre plates to act as negative controls.

Table 4 List of cultivated varieties used for resistance testing to AWMV, SBCMV, SBWMV and WSSMV. Where available, information on previously reported field reactions is included; (R) resistant; (S) susceptible; (N) not previously tested.

Cultivar	Resistance reaction			
	SBCMV	WSSMV	SBWMV	AWMV
Cadenza	R[2]	N	N	N
Charger	R[12]	R[1]	N	N
Claire	R[12]	R[1]	N	N
Coker 9663	N	S[3]	S[3]	N
Deben	S[12]	R[1]	N	N
Ernie	N	R[3]	S[3]	N
Hawk	N	R[3]	R[3]	N
Jagger	N	R[3]	R[3]	N
Sierra	N	S[3]	R[3]	N
Tam 110	N	S[3]	S[3]	N
Tanker	S[12]	R[1]	N	N
Tremie	R[2]	N	N	R[5]
Xi 19	R[12]	R[1]	N	N

[1] Budge *et al* (2002)
[2] Bayles and Napier (2002)
[3] Armitage *et al*. (1990)
[4] Bob Hunger personal communication
[5] Hariri *et al* (2001)

Results

Germination of seed for cultivars Charger, Tremie and Tanker was inconsistent and so these cultivars were removed from the experiment. All root material tested negative for AWMV, suggesting the absence of infectious virus in the soil sample collected from France. The majority of the leaf material tested negative for SBCMV and SBWMV, however sporadic positive results were recorded for susceptible cultivars for both viruses (data not shown).

Cultivars known to be susceptible to SBCMV and SBWMV had consistently higher A_{405} values than cultivars known to be resistant to either virus (Table 5). When A_{405} values from the SBCMV specific TAS ELISA were plotted against those for the SBWMV ELISA, distinct clusters of resistant and susceptible cultivars were observed (Figure 13). A_{405} values for WSSMV in cultivars designated susceptible to the virus were not consistently higher than those for cultivars deemed resistant to WSSMV (data not shown).

Table 5 Comparison of mean A_{405} values (from four replicate modules) from ELISA tests for roots grown in soil containing SBCMV (UK) or SBWMV and WSSMV (USA). Corresponding standard error is displayed in parenthesis. Each soil was run in an artificial immersion system placed within a CE room. Where available, information on previously reported field reactions is included; (R)

resistant; (S) susceptible; (N) not previously tested. Healthy control represents the mean of 6 replicates of healthy roots from cv. Riband.

Cultivar	**Average A_{405} values**	
	SBCMV	**SBWMV**
Cadenza	^{R}0.083 (0.083)	^{N}0.114 (0.023)
Claire	^{R}0.092 (0.009)	^{N}0.080 (0.008)
Coker 9663	^{N}0.254 (0.089)	^{S}1.532 (0.519)
Deben	^{S}0.505 (0.198)	^{N}1.851 (0.518)
Ernie	^{N}0.340 (0.162)	^{S}1.693 (0.511)
Hawk	^{N}0.094 (0.009)	^{R}0.251 (0.166)
Jagger	^{N}0.105 (0.009)	^{R}0.133 (0.047)
Sierra	^{N}0.126 (0.026)	^{R}0.091 (0.013)
Tam 110	^{N}0.524 (0.316)	^{S}2.367 (0.112)
Xi 19	^{R}0.081 (0.004)	^{N}0.133 (0.030)
Healthy control	0.069 (0.003)	0.074 (0.004)

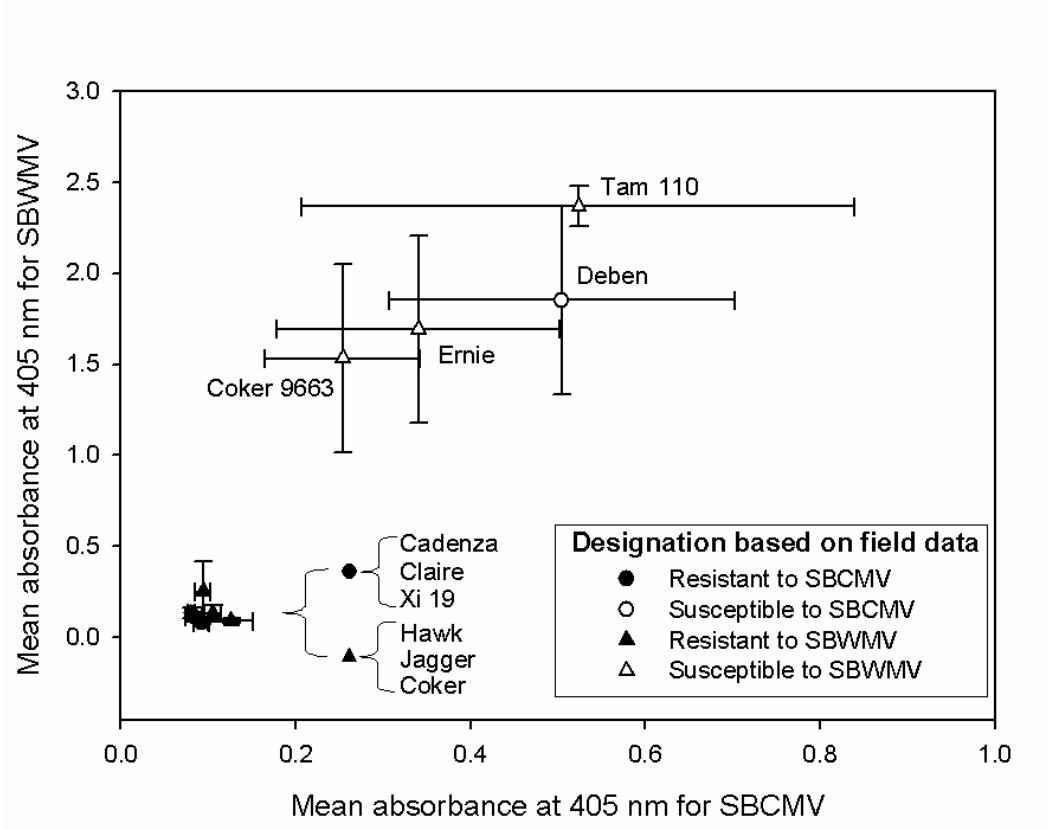


Figure 13 Average absorbance values (A_{405}) with corresponding standard errors from TAS ELISA tests for SBCMV and SBWMV for the roots of 10 cultivars of winter wheat grown using the CE test at 15°C for 18 weeks. Cultivars are designated resistant or susceptible to each virus based on field studies (Bayles and Napier 2002, Budge *et al*. 2002; Armitage *et al* 1990; Bob Hunger personal communication). Clear resistant (bottom left) and susceptible (top right) clusters are formed for both viruses suggesting resistance to one virus will function to protect against the other virus.

Discussion

This work has clearly demonstrated that a CE test developed for phenotyping SBCMV resistance is also suitable for profiling cultivar resistance to SBWMV. The conditions in this experiment differed slightly to allow for the higher temperature requirement and longer incubation necessary for AWMV infection. However, data from earlier in the project suggested longer incubation times at a temperature of 15°C gave similar results to an 8-week incubation at 12°C. These data suggest the resistance mechanisms for both viruses are likely to be functioning in a similar way. Publications looking at the biology or resistance of SBWMV could provide useful insight into the resistance of SBCMV. For example, two resistance genes were identified in Brazilian cultivars of *Triticum*

aestivum using screening of backcross populations derived from resistant and susceptible parents (Barbosa *et al*.2006).

The lack of results for AWMV was disappointing given the resources involved in collecting the soil and testing the wheat lines. The main reason for extending the test to 14 weeks was to follow the protocol for generating infecting wheat with AWMV as stated in Hariri *et al*. (2001). The most likely explanation for this negative result is that the soil provided to CSL did not contain AWMV.

It was found that the A_{405} values for WSSMV in cultivars designated susceptible to the virus were not consistently higher than those for cultivars deemed resistant to WSSMV suggesting the CE test is not suitable for discriminating wheat lines which are resistant or susceptible to WSSMV. It also suggests that the resistance mechanism in WSSMV is most probably functioning in a different way to that seen for SBCMV and SBWMV. Resistance to WSSMV is unlikely to act by limiting the movement or replication of the virus in root tissue. Cadle-Davidson *et al*, (2006a; 2006b) investigated the field resistance of small grain genotypes SBWMV and WSSMV. Cultivars showing resistance to WSSMV did not necessarily show resistance to SBWMV. In addition, UK cultivars known to have resistance to SBCMV proved, to be resistant to WSSMV in field trials in France and Italy, again supporting the data in the current study (Budge *et al*., 2002).

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APPENDIX 1

Clone name	Approx. size of original product from initial screen	sequence file name	Size of sequence	Similarity using Blastn database	Similarity using TIGR database	Similarity using Blastx database	Predicted peptides identified by Genscan
C-sub A9	250	C sub A9	234	42bp region similar to oryza sativa predicted mRNA-gibberellin responsive genes;	None	ZmGR2a [Zea mays] gibberellin responsive gene, 42/93 identities	MKLKFQSRLLPKLTLQLKKKNGX and XQAVEEIQAVVAAVQQQETTGDETQVP VEAAAETDAPAQEEKRE
C-sub E3	180	C sub E3	149	none	38bp region similar to Photosystem I P700 apoprotein A1; also 61/93 (65%) CK152227 homologue to GP|10800865|emb beta-1 4-endoglucanase 1 precursor {Heterodera schachtii}	none	none
C-sub G8	220	C sub G8	122	none	50bp match to Plasma membrane intrinsic protein (aquaporin)	none	none
C-sub F2	450	C sub F2 b	485	263bp match to cDNA clone CA733072	None	endo-1,4-beta-glucanase (hydrolyze polysaccharides) 21/64 identities	none
		C sub F2 c	628	none	None	hypothetical protein [Escherichia coli] Identities = 19/34	MIVNGLCRTSRTDEGVHRTQPSVRDER VGEERKNTDNTAVFRVLAHKLRIAAHVL SGSIS
		C sub F2 d	225	none	91bp region similar to an unknown protein in Tigr wheat library	hepatocellular carcinoma-associated antigen HCA108 Identities = 13/25	MAAAGITFRGAARAX

		C sub F2 e	319	none	None	none	ASWWYCTLQQWPFRGNVARTRSRDFL IPAWLPSKKHTVYVSEIIVKKDRFRCRTL ILWTAPNGPTE
C-sub D5	180	C sub D5 a	116	none	114bp region similar to ATP synthase F0 subunit	none	none
		C sub D5 b	176	none	142bp region similar to an unknown protein in Tigr wheat library	none	VANGSVLRLIKRCTVRPGQLTGVRHX
		C sub D5 c	340	none	139bp region similar to a hsp70	Transketolase Identities = 17/54 (an enzyme involved inthe Calvin Cycle)	none
C-sub E8	170	C sub E8 a	86	18bp identical to ribosomal genes	48/50 identity to Aquaporin	none	none
		C sub E8 b	148	none	none	none	none
		C sub E8 c	86	none/	48/50 identity to Aquaporin	none	none
		C sub E8 d	125	86/124 identity to another wheat clone CA621587	none	none	MLDNMKGKNEIHILVPNLLRICFVAPQP
C-sub A1	450	C sub A1 a	1109	42bp region similar to Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 2	201bp identical to another wheat clone CA733072	hypothetical proteins; Heat shock protein DNAJ bacteria Identities = 17/48; putative transporter, permease protein bacteria Identities = 20/56	XEIENTEPVEVEENRSASDLQAEEVKET EAINERTVANTSELPEDVTSNVVPADEA APEEHVIATEATVDIPEAQGPELEETKST CPGGRSPSLGNSRPPARR
		C sub A1 b	558	weakly similar (~ 60%) to numerous beta-galactosidase genes, but could be vector	none	beta-galactosidase [Cloning vector pUG7]	none
		C sub A1 c	520	98bp region similar to Homo sapiens DNA; gamma gene loci of immunoglobulin heavy chain (91% identity)	179bp similar to 22kD alpha zein 1 (Zea mays) ref CA617194	oligoendopeptidase F, putative Identities = 12/42	none

C-sub B3	450	C sub B3	430	42bp region similar to Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 2, complete sequence/	92% similarity (201/217) to another wheat clone, CA733072	Hypothetical protein CBG01887 [Caenorhabditis briggsae] Identities = 36/134; AAA ATPase Identities = 34/139	XEEPHAEQVTVTERTVDIPQAQEPELEEIENTEPVEVEENRSASDLQAEEVKETEAINER
C-sub C5	190	C sub C5 a	123	none	96% similarity (48/50) to Aquaporin	none	none
C-sub C7	180	C sub C7 a	118	none	weakly similar to human interleukin-1 receptor-associated kinase I	none	none
		C sub C7 b	229	none	none	NADH dehydrogenase subunit 5 Identities = 14/37	none
		C sub C7 c	150	none	70% similarity (61/86) to another wheat clone, ref: CA600533	none	none
C-sub D4	450	C sub D4	125	none	73% similarity (36/49) to photosystem I P700 apoprotein A1 (Anthoceros punctatus)	none	none
C-sub D7	190	C sub D7 a	389	none	none	conserved hypothetical protein [Yersinia pestis Identities = 33/79	none
		C sub D7 b	73	none	96% similarity (48/50) to Aquaporin	none	none
C-sub F10	180	C sub F10	153	none	90% similarity (36/40) and 100% similarity (23/23) to Photosystem I P700 apoprotein	none	none

C-sub G1	450	C sub G1	430	42bp region similar to Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 2, complete sequence	92% similarity (201/217) to another wheat clone, CA733072	Hypothetical protein Identities = 36/134; AAA ATPase Identities = 34/139	XEEPHAEQVTVTERTVDIPQAQEPELEE IENTEPVEVEENRSASDLQAEEVKETEA INER
C-sub H11	180	C sub H11	156	none	90% similarity (36/40) and 100% similarity (23/23) to Photosystem I P700 apoprotein	none	none
I-sub A4	180	I sub A4 b	115	none	96% similarity (76/79) to Hordeum jubatum 18S rRNA gene. The same region also has 96% similarity to Histone H3-like protein (ref: TC233134 tigr database)	none	none
		I sub A4 a	213	none	none	none	none
		I sub A4 c	258	none	66% similarity (120/180) to another wheat clone, ref CA624204	unnamed protein product [Mus musculus] Identities = 15/56; similar to FERM domain containing protein 6 Identities = 15/56	none
I-sub B3	210	I sub B3 a	162	none	none	none	none
		I sub B3 b	240	none	100% similarity (130/130) to 14-3-3 protein	none	none
		I sub B3 c	161	none	none	none	MRINRISDFRNVPGTNCDPYIQMIALDN TX
I-sub B7	150	I sub B7	159	none	96% similarity (58/59) to Mini-chromosome maintenance 7	none	none
I-sub C10	150	I sub C10	160	none	98% similarity (78/79) to Histone H3-like protein	none	none

I-sub A3	210	I-sub A3	206	55bp region aligns to Hordeum vulgare mRNA for 14-3-3 protein (Hv1433c) proteins expressed in barley leaves 14-3-3 proteins appear to effect intracellular signalling	100% similarity (130/130) to 14-3-3 protein	none	none
I-sub A11	200	I-sub A11a	245	28bp region Aeromonas hydrophila alpha-hemolysinUpon binding, the monomers oligomerize to form a water-filled transmembrane channel that facilitates uncontrolled permeation of water, ions, and small organic molecules. Rapid discharge of vital molecules, such as ATP, dissipation of the membrane potential and ionic gradients, and irreversible osmotic swelling leading to the cell wall rupture (lysis), can cause death of the host cell.	none	none	CSAVTISRFLPITQTDSYRYAQRRLT
		I sub A11b	118	37 bp region similar to Stylonychia lemnae macronuclear development protein 24	96% similarity (57/59) to another wheat clone (CK197452)	none	none

		I sub A11c	221	25bp region aligns with many sequences but no information on function	60% similarity (74/123) to another wheat clone, CV064749	unnamed protein product [Aspergillus oryzae] Identities = 22/62 this protien in turn is similar to phosphorylase (catalyzes the production of glucose)	none
C1 sub B6	150	C1 sub B6	119	none	none	none	none
C1 sub E6	140	C1 sub E6	154	none	none	none	none
C2 sub G3	220	C2 sub G3	274	none	93% identity (231/248) to ZmGR2b (gibberellin responsive) protein	none	XQAVEEIQAVVAAVQQQETTGDETQVP VEAAAETDAPAQEEKRE
I sub D1	350	I sub D1	289	100% similarity to 18S gene of multiple species	74% similarity to 18S rDNA gene for multiple species	none	none
C1 sub D12	250	C1 sub D12	274	none	93% identity (231/248) to ZmGR2b (gibberellin responsive) protein	none	none
C1 sub G4	420	C1 sub G4	464	none	aligns with an EST similar to a putative secreted protein from Streptomyces coelicolor.	none	none
C1 sub E9	400	C1 sub E9	497	none	aligns with an EST similar to a putative secreted protein from Streptomyces coelicolor.	none	none