Project title:	Genetics of resistance to Verticillium wilt in strawberry
Project number:	SF 135
Project leader:	Dr David Simpson, East Malling Research
Report:	Final, March 2014
Previous report:	Interim, March 2013
Key staff:	Dr David Simpson Dr Richard Harrison Ms Abigail Johnson Ms Fiona Wilson Ms Kirsty McLeary
Location of project:	East Malling Research, Kent
Industry Representative:	Richard Stanley
Date project commenced:	1 April 2012
Date project completed (or expected completion date):	31 March 2014

DISCLAIMER

AHDB, operating through its HDC division seeks to ensure that the information contained within this document is accurate at the time of printing. No warranty is given in respect thereof and, to the maximum extent permitted by law the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

[Name] Dr David Simpson
[Position] Research Leader, Genetics and Crop Improvement
[Organisation] East Malling Research

Signature ..... Date .....

[Name]
[Position]
[Organisation]

Signature ..... Date .....

Report authorised by:

[Name]
[Position]
[Organisation]

Signature ..... Date .....

[Name]
[Position]
[Organisation]

Signature ..... Date .....

CONTENTS

GROWER SUMMARY

Headline

DNA markers for resistance to Verticillium wilt found to be present in a wide range of cultivars and other accessions available to UK strawberry breeders

Background and expected deliverables

Genes responsible for resistance to Verticillium wilt had been identified in an earlier project funded by the Biotechnology and Biological Sciences Research Council (BBSRC). The primary aim of this project was to validate markers for these resistance genes by looking for their presence or absence in a wide range of unrelated strawberry cultivars and correlating this with the field resistance of those cultivars to *Verticillium dahliae*. A second aim was to investigate if there was a distinction between resistance and tolerance to *V. dahliae* and if these were under separate genetic control. Once validated, the resistance gene markers will enable plant breeders to undertake marker-assisted breeding in strawberry, which will benefit the industry through the development of cultivars with strong and durable resistance to Verticillium wilt.

Summary of the project and main conclusions

In an earlier project, genes associated with resistance to Verticillium wilt had been identified in a progeny of 188 seedlings from the cross Redgauntlet x Hapil. We tested for the presence of molecular markers for five of these genes in 46 cultivars and breeding lines that had all previously been characterised for resistance to wilt on multiple occasions, using a highly infested field plot maintained at EMR. The majority of these lines were either unrelated or only distantly related to Redgauntlet, which was the source of resistance in the original cross. All of the markers were found to be more common among the resistant lines than the susceptibles in the 46 lines that formed the validation set but no single marker could be considered to be an accurate predictor of resistance when considered in isolation.

Following the validation of the markers, a further 128 strawberry lines were tested for presence of the resistance markers. These lines included modern cultivars, old cultivars and breeding lines from the EMR collection, and selections and parental lines being evaluated by the EMR Strawberry Breeding Club. In this wider collection the frequency of the alleles was variable and two, $HRGP_{213}$ and RVd4, were found to be rare in modern cultivars but

more common in the old cultivars contained in the EMR collection. Cultivars and lines with the strongest field resistance to wilt typically had two or more different resistance markers present. The markers have been used in planning the 2013 and 2014 crossing programmes for the EMR Strawberry Breeding Club. Crosses were designed that will increase the probability of resistant individuals being present in the seedling populations that will be evaluated in 2014 and 2015.

A glasshouse experiment comparing two isolates of Verticillium wilt showed a large difference in their pathogenicity. Plants inoculated with the more pathogenic isolate were used to quantify the amount of pathogen DNA in petioles from a range of strawberry lines including resistant, susceptible and intermediate types. *Verticillium dahliae* DNA was found to be present in all lines except one, suggesting that it is not possible to distinguish between resistance and tolerance to wilt but that it is a straightforward quantitative trait where multiple genes have a cumulative effect on the level of resistance expressed.

Financial benefits

There will be downstream benefits from this project as the resistance markers will enable the EMR Strawberry Breeding Club and other UK breeders to develop future cultivars with strong and durable resistance to Verticillium wilt. These cultivars will benefit growers who wish to grow in the soil rather than substrate and will reduce the need for soil fumigation. Soil production is a cheaper system than substrate growing on table tops since the capital investment is not required for the table-top system and there is no requirement to purchase substrate. Harvesting costs are lower on table tops but this does not fully compensate for the increased expenditure in other areas.

Action points for growers

There are no immediate action points for growers but they should be aware of the opportunities offered by future cultivars with strong resistance to Verticillium wilt.

SCIENCE SECTION

Introduction

The project BB/E007074/1 'A genetic system to study resistance to the soil-borne pathogen *Verticillium dahliae* in strawberry' was funded at EMR by BBSRC from October 2007 to September 2011. One of the main objectives of this project was to develop a genetic linkage map for a strawberry progeny segregating for resistance to Verticillium wilt (Redgauntlet x Hapil, acronym RGxH) and locate quantitative trait loci (QTL) that are contributing to resistance found in Redgauntlet. Wilt resistance is a continuous trait with several genes contributing to the expression in an additive way, these are known as QTL. This objective was successful and identified molecular markers for three significant resistance QTL. The intention of this project was to validate the resistance markers before using them in the breeding programme for the East Malling Strawberry Breeding Club (EMSBC), of which HDC is a member. It is essential to validate the resistance markers to determine if they are associated with resistance in a wide range of cultivars and breeding lines, not just those related to Redgauntlet.

Another objective of this project was to develop a molecular method to attempt to distinguish between resistance and tolerance to *V. dahliae* by measuring the amount of pathogen DNA in the petioles of infected plants. Several cultivars that are classified as resistant are very vigorous when grown in the absence of the pathogen and it was considered likely that these are tolerant i.e. the vigorous root growth is able to tolerate a level of fungal infection without the plant developing symptoms. The plan was to quantify the pathogen DNA present in a range of plants showing different symptom expression. If clear differences were found then this would permit classification of the existing QTL for resistance or tolerance and may also identify additional QTL.

Materials and methods

DNA extraction from strawberry genotypes chosen for marker validation

During the spring and summer of 2012, newly-emerged, unexpanded leaves were collected from actively growing plants of 99 EMSBC selections, breeding lines and parental cultivars, 30 modern commercial cultivars (including six proprietary lines from Driscoll's Genetics Ltd and three from Edward Vinson Ltd) and 45 accessions from the EMR collection (174 different lines in total). Leaves were snap frozen in liquid nitrogen and stored in a freezer at -80^{o}C until required for DNA extraction. The frozen leaf tissue was ground to a fine powder

using ball-bearings in a mixer-mill and high-quality DNA was then extracted using the commercial DNeasy mini-kit manufactured by Qiagen.

Molecular marker screening and validation

The ABI genotyping platform was used with fluorescently labelled primers to screen for the presence of five resistance markers in the DNA samples from the 174 lines. The presence or absence of specific alleles at the resistance marker loci was recorded for each individual line.

Glasshouse screening of progeny segregating for resistance to Verticillium wilt

Year 1

Twenty two seedlings from the RG×H progeny were selected based on their differential response to field infection with *V. dahliae*, as observed in an earlier project (BB/E007074/1). Fifteen plants from each of these 22 lines, along with Redgauntlet and Hapil, were clonally propagated by pinning down runners into sandy compost in 9 cm pots. When 10-12 weeks old, the plants were removed from their pots, and the compost washed from their roots. The roots were then trimmed to a standard length of 70mm to wound the plant and each plant had the roots immersed for 15 minutes in a wilt suspension of 10^6 conidia per ml. Plants were re-potted into 1 L pots and placed in three randomised blocks in a glasshouse with 16h day and temperature of 22^{o}C day, 14^{o}C night. Individual dripper lines were used for irrigation to allow careful control of water availability, which was necessary due to differences in vigour between the seedlings. Five treatments were applied, three individual *V. dahliae* isolates taken from different host cultivars, one mixed isolate and a control with no pathogen. The plants were arranged in a randomised block design with three replicates.

Plants were observed at weekly intervals for 9 weeks and any symptoms of wilting were recorded. At the end of the experiment petiole tissue was harvested from all plants and snap frozen in liquid nitrogen.

Year 2

Thirty seven seedlings from the RG×H progeny were selected, based on the presence of different numbers of markers for resistance QTL. The progeny parents, Redgauntlet and Hapil, were also included along with seven unrelated genotypes that had been found to show strong field resistance in earlier tests. Plant propagation, cultivation and inoculation procedures were the same as in Year 1. A randomised block design with three replicates was used again but in this case more genotypes were included but only three treatments

were applied, two individual *V. dahliae* isolates and a control with no pathogen. One isolate had also been used in Year 1, while the second had been isolated from a cultivar unrelated to the RG×H progeny. Plants were observed at weekly intervals for 12 weeks and any symptoms of wilting were recorded, resulting in a cumulative score at the end of the experiment, where a higher value indicated increased susceptibility. Petioles were harvested on 9 September 2013 (T'0'), immediately before inoculation of the plants, and on 22 October 2013 (T'1'), frozen in liquid nitrogen and stored at -80 ^{0}C. DNA was extracted using the same method as for leaf tissue, described above.

Quantitative real-time PCR to determine levels of pathogen in plant tissue

Primers specific to the strawberry gene elongation factor 1- α (FaEF1α) were used to quantify strawberry DNA and *V. dahliae* specific primers for β-tubulin (VertBt) were used to quantify *V. dahliae* DNA (Amil-Ruiz et al. 2013). Real time PCR was performed using the Applied Biosystems 7500 Real Time PCR System. For each plant replicate, qRT-PCR for T'0' and T'1' DNA samples was performed on the same 96-well plate. FaEF1α and VertBt amplifications were performed in separate reactions for each DNA sample, each reaction was replicated three times.

Relative quantification of DNA was calculated by the deriving the ratio (ΔCt) between the amounts (the Ct value) of the target gene (VertBt) and the endogenous reference gene (FaEF1α) (Atallah et al. 2007). The Ct value is defined as the cycle in which there is a significant increase in fluorescence (linked to the amount of amplified DNA), above the threshold. The threshold is the level of fluorescence above the baseline at which the signal can be considered not to be background. The Ct value is consequently related to the initial amount of DNA and is in inverse proportion to the expression level of the gene, i.e. if the Ct value is low it means the fluorescence crosses the threshold early, meaning that the amount of target in the sample is high.

For each sample the ΔCt between the target gene and the reference gene was calculated for the T'1' samples and T'0' samples. ΔCt = Cttarget – Ctreference gene. The mean ΔCt values were derived and the difference between the ΔCt of the T'1' and T'0' reactions was calculated for each sample, giving the ΔΔCt value: ΔΔCt = (Cttarget – Ctreference) T'0' – (Cttarget – Ctreference) T'1'.

Results

Marker validation

In Year 1 the work focused on validation of the strongest quantitative trait locus (QTL) for wilt resistance that had been identified in the RGxH progeny. A gene coding for hydroxyproline-rich glycoprotein (HRGP) had been found to be underlying this QTL. HGRP proteins are known to be associated with plant defence, making this a likely candidate gene for resistance to wilt and suitable for use as a marker. In the BBSRC project five alleles for this HGRP gene had been identified, with two associated with resistance inherited from Redgauntlet. Initial analyses had suggested that both alleles 213 and 232 were associated with resistance but subsequently, after more markers had been added to the linkage map, it was found that allele 213 was most closely linked to the resistance QTL. However, results for both HRGP alleles are presented in the tables below. During Year 1 an additional allele of HRGP (237) was found to be expressed in germplasm unrelated to RGxH.

In Year 2, three further resistance markers derived from Redgauntlet were investigated. RVd7, RVd6 and RVd4. Two of these had been identified in the BBSRC project while the third was for an additional resistance QTL that had been discovered during further analysis of the linkage map and phenotypic data during Year 1.

The validation of the markers involved genotyping a set of 46 cultivars and breeding lines that had previously been characterised for resistance to wilt on multiple occasions, using a highly infested field plot maintained at EMR and containing a mixture of isolates of *V. dahliae*. This had provided robust phenotypic data for these 46 lines and, importantly, the majority of them were either unrelated or only distantly related to Redgauntlet.

The results are summarised in Table 1. Overall, the markers RVd7, $HRGP_{232}$, $HRGP_{213}$, RVd6 and RVd4 are present in 34%, 36%,13%, 50% and 19% of the 46 lines, respectively, but in all cases they are over represented in the resistant category, with the corresponding percentages being 40%, 65%, 20%, 70% and 30%. Table 1 shows that there is a clear trend that the markers are more frequently present in the resistant and intermediate material than in the susceptible lines. However, the picture is not straightforward. For example, only $HRGP_{213}$ was completely absent in the 18 susceptible lines, but this is the least common of all the markers, being present in only 13% of the lines overall. The marker RVd6 was the most common overall and present in 70% of the resistant lines but it was also detected in 27% of the susceptibles.

Table 1. Presence (1) or absence of 5 resistance markers in 46 cultivars and breeding lines

	Marker					
Cultivar or line	RVd7	$HRGP_{232}$	$HRGP_{213}$	RVd6	RVd4	**Class†**
Elsanta	1					Sus
EM0881				1		Sus
EM1442				1		Sus
EM1580						Sus
EM1733	1			1		Sus
EM1772						Sus
EM1812						Sus
EM1856						Sus
EM1871				1		Sus
EM1942						Sus
EM1966						Sus
Emily					1	Sus
Eros	1					Sus
Hapil						Sus
Holiday					1	Sus
Mae						Sus
Malling Centenary	1	1		1		Sus
Vibrant						Sus
Cambridge	1		1			Int
Elegance		1				Int
EM1399				1		Int
EM1500	1			1	1	Int
EM1727				1		Int
EM1792	1	1	1	1		Int
Malling Pearl		1				Int
Symphony	1					Int
Albion	1	1				Res
Alice	1	1		1		Res
Cupid				1		Res
EM0555	1	1	1			Res
EM0701				1	1	Res
EM0972					1	Res
EM1624		1	1	1		Res
EM1677						Res
EM1682	1				1	Res
EM1785		1		1		Res
Everest		1		1		Res
Fenella				1	1	Res
Finesse		1		1		Res
Flamenco	1	1		1		Res
Florence		1		1		Res
Judibell			1	1	1	Res
Pegasus	1	1		1		Res
Redgauntlet	1	1	1	1	1	Res
Senga Sengana		1		1		Res
Sonata	1	1				Res

†Classification based on field test. Res=resistant, Int=intermediate, Sus=susceptible

Screening cultivars, selections and breeding lines for presence of the five markers

Following the initial validation of the markers in the 46 lines, a further 128 lines were screened to determine which had any of the resistance markers present. The results for all 174 lines are shown in Tables 2-4, with the lines categorized as modern cultivars, accessions from the EMR collection and current breeding lines for the Strawberry Breeding Club (SBC).

Table 2. Presence of five markers associated with resistance to *V. dahliae* in 30 modern strawberry cultivars

RVd7	HRGP232	HRGP213	RVd6	RVd4	None
Buddy	Buddy	Amelia	Amelia	Amelia	Driscoll 5
Camarillo	Driscoll 2	Judibell	Buddy	Fenella	Sweetheart
Driscoll 2	Driscoll 3		Cupid	Flair	Vibrant
Driscoll 3	Driscoll 4		Driscoll 1	Judibell	
Driscoll 4	Elegance		Driscoll 2	Malwina	
Elsanta	Elianny		Elianny		
Eves Delight	Evie2		Evie2		
Evie2	Finesse		Fenella		
M Centenary	Florina		Finesse		
Sonata	M Centenary		Judibell		
Sweet Eve	Sonata		Malwina		
Symphony			M Centenary		
Verity			Portola		
			Rumba		

Table 3. Presence of five markers associated with resistance to *V. dahliae* in 48 accessions from the EMR strawberry germplasm collection

RVd7	HRGP232	HRGP213	RVd6	RVd4	None
Alice	Aberdeen	Addie	Addie	Aberdeen	Delmarvel
C Favourite	Addie	Allstar	Alice	Addie	EM1108
Christine	Alice	C Favourite	EM0701	Earliglow	Hapil
Darselect	EM0555	Christine	EM0791	EM0701	Mae
EM0555	EMR314	EM0555	EM0881	EM0972	Rainier
EM1113	Korona	Korona	EM1399	EM1500	
EM1500	Little Scarlet	Osmanli	EM1442	Emily	
Eros	Mara d Bois	Pandora	EM1500	Holiday	
Mara d Bois	Mimek	Redgauntlet	Korona	Mara d Bois	
Merton Dawn	Nyoho	R Sovereign	Little Scarlet	Nyoho	
Nyoho	Osmanli	Sierra	Merton Dawn	Osmanli	
Pandora	Pegasus		Osmanli	Pandora	
Pegasus	P de Prague		Pandora	Redgauntlet	
P de Prague	Providence		Pegasus	R Sovereign	
Redgauntlet	Redgauntlet		Perfection	Sierra	
R Sovereign	R Sovereign		Providence		
Sierra	S Sengana		Redgauntlet		
	Sierra		R Sovereign		
	Tribute		S Sengana		
			Sierra		
			Siletz		
			Tribute		
			Wiltguard		

Table 4. Presence of five markers associated with resistance to *V. dahliae* in 99 selections, breeding lines and cultivars being used as parents by the EMR Strawberry Breeding Club.

RVd7	HRGP232	HRGP213	RVd6	RVd 4	None
Albion	Alba	Argentera	Alba	Argentera	EM1580
Charlotte	Albion	EM1620	Charlotte	EM1682	EM1677
DNBL205	Argentera	EM1624	Diamante	EM1775	EM1746
DNBL212	Charlotte	EM1786	DNBL205	EM1786	EM1752
EM1682	Clery	EM1792	DNBL212	EM2065	EM1772
EM1733	Diamante	EM1905	EM1607	EM2085	EM1781
EM1756	EM1620	EM1949	EM1620	EM2090	EM1812
EM1792	EM1624	EM2053	EM1624	EM2092	EM1839
EM1832	EM1704	EM2087	EM1704	EM2156	EM1856
EM1837	EM1785	EM2090	EM1727	EM2182	EM1942
EM1977	EM1792	EMR474	EM1733	Granda	EM1966
EM1998	EM1795	EMR485	EM1775	I 91-136-1	EM2161
EM2053	EM1832	EMR489	EM1785	Marmolada	EM2181
EM2062	EM1860	I 91-136-1	EM1786	P82	EMR506
EM2065	EM2034	I 94-153-51	EM1792	P85	
EM2087	EM2056	P82	EM1832	SDBL120	
EM2150	EM2062	P85	EM1871		
EMR348	EM2064		EM1888		
EMR349	EM2065		EM1905		
EMR437	EM2066		EM2056		
EMR466	EM2090		EM2062		
EMR470	EM2092		EM2064		
EMR474	EM2157		EM2090		
EMR477	EM2170		EM2131		
EMR489	EMR348		EM2134		
EMR521	EMR437		EM2135		
EMR537	EMR466		EMR349		
EMR555	EMR474		EMR437		
EMR556	EMR485		EMR477		
EMR562	EMR489		EMR489		
EMR564	EMR521		EMR555		
EMR565	EMR537		EMR556		
EMR568	EMR562		EMR562		
EMR583	EMR565		EMR564		
Flamenco	EMR569		EMR565		
Marmolada	Everest		EMR568		
SDBL101	Flamenco		EMR574		
	Florence		EMR590		
	Malling Pearl		Everest		
	Marmolada		Flamenco		
			Florence		
			Granda		
			I 91-136-1		
			I 94-153-51		
			Marmolada		
			P82		
			SDBL101		
			SDBL122		
			SDBL123		
			SDBL126		

Table 5. Percentage of plants with each of the five markers present in four germplasm sets

Germplasm	Marker RVd7	HRGP232	HRGP213	RVd6	RVd4
Modern Cvs	**43**	**40**	**6**	**46**	**16**
Collection	**37**	**42**	**24**	**51**	**33**
SBC lines	**37**	**40**	**17**	**50**	**16**
Validation Set	***34***	***36***	***13***	***50***	***19***

Table 5 summarises the information in Tables 2-4 and shows the percentages of plants with each marker present in the three different germplasm sets. The distribution in the 46 lines used for validation, which includes lines from all three sets, is included for comparison. The two most common markers, $HRGP_{232}$ and RVd6, show very little variation. $HRGP_{213}$, which was the least common marker in the validation set is rare in modern cultivars but much more common in the older germplasm in the collection and relatively more common in the germplasm being used by the East Malling Strawberry Breeding Club. RVd7 is slightly over represented in the modern cultivars whereas RVd4 is twice as common in the older germplasm of the collection as it is in the modern cultivars or the EMSBC germplasm.

The maximum number of markers present among the modern cultivars was three, found in six cultivars, namely Amelia, Buddy, Driscoll 2, Evie2, Judibell and Malling Centenary. Driscoll 2 is a numbered selection that has not yet been released. In the EMR collection the cultivars Redgauntlet, Royal Sovereign and Sierra each had all five markers present. This was expected for Redgauntlet, as it was the resistant parent of the mapping progeny. Royal Sovereign in a 19th century British cultivar and Sierra is from California but has not been in commercial production for over 50 years.

The maximum number of markers in the lines currently being used by the East Malling Strawberry Breeding Club was four, in EM1792, EM2090, EMR489 and the Italian cultivar Marmolada (aka Onebor). EMR489 is an everbearing selection that has progressed to advanced trials, whereas EM1792 and EM2090 are breeding lines.

Glasshouse screening of progeny segregating for resistance to Verticillium wilt

Despite using a well-tested protocol for infecting plants with *V. dahliae*, the symptom expression in the Year 1 experiment was very low. Of 288 plants inoculated, only 61 (21%) developed visible symptoms of wilt (Table 6) and in many cases these symptoms were mild. Even with Hapil, the susceptible standard, only 33% of the plants developed symptoms.

This was a surprising and disappointing result. The start date for the experiment had to be delayed due to other glasshouse experiments overrunning during the summer as a consequence of the cool weather. It was originally intended to inoculate the plants in early September but this was delayed until 10 October. Although the plants were kept in a warm glasshouse with lighting to provide 16 h days, they were clearly growing less actively than expected and it is possible that they consequently did not develop the typical symptoms of wilt. The original intention had been to take petiole samples for DNA extraction when 75% of the Hapil plants were showing symptoms. This point was never reached so the samples were taken after nine weeks, when the experiment was terminated.

In Year 2, for the second experiment, plants were inoculated over four weeks earlier, on 9 September and on this occasion 30% developed clear wilt symptoms and a further 13% were unhealthy but did not display the clear symptoms typical of wilt. Approximately 28% of the plants did not appear to recover from the process of root trimming and re-potting and died, usually during the four weeks following inoculation. Because these plants did not survive long enough to display clear symptoms, it was not possible to determine if they had died as a result of the wilt infection.

The experiment in Year 1 did not show any clear differences in pathogenicity between the isolates but in Year 2 isolate 12253 (which was used in both years) was found to be significantly more pathogenic than isolate 12158 ($p<0.001$, Table 7). There was also a significant genotype x isolate interaction ($p=0.02$) and a small number of individuals had a higher mean wilt score for 12158 than for 12253. The wilt scores for the two isolates were not significantly correlated.

Table 6. Plants developing symptoms of wilt among 24 genotypes of strawberry inoculated with three single isolates and one mixed isolate of *V. dahliae.* Three replicates per treatment combination

Genotype	***V. dahliae* isolate**				
	12251	**12252**	**12253**	**Mixed**	**Total**
Hapil	1	1	2	0	4
Redgauntlet	0	0	0	0	0
RH002	0	0	1	0	1
RH003	0	1	0	1	2
RH017	0	0	0	1	1
RH018	1	0	1	0	2
RH020	0	0	0	1	1
RH032	1	3	1	1	6
RH034	0	0	3	1	4
RH035	0	1	2	0	3
RH043	1	0	1	1	3
RH047	1	2	1	1	5
RH065	0	1	0	1	2
RH075	0	2	0	1	3
RH076	0	1	0	0	1
RH079	1	0	1	0	2
RH088	3	1	0	1	5
RH101	0	0	0	1	1
RH104	1	1	2	0	4
RH117	1	1	1	0	3
RH118	0	1	0	0	1
RH120	1	2	1	0	4
RH172	1	0	2	0	3
RH188	0	0	0	0	0
Total	13	18	19	11	61

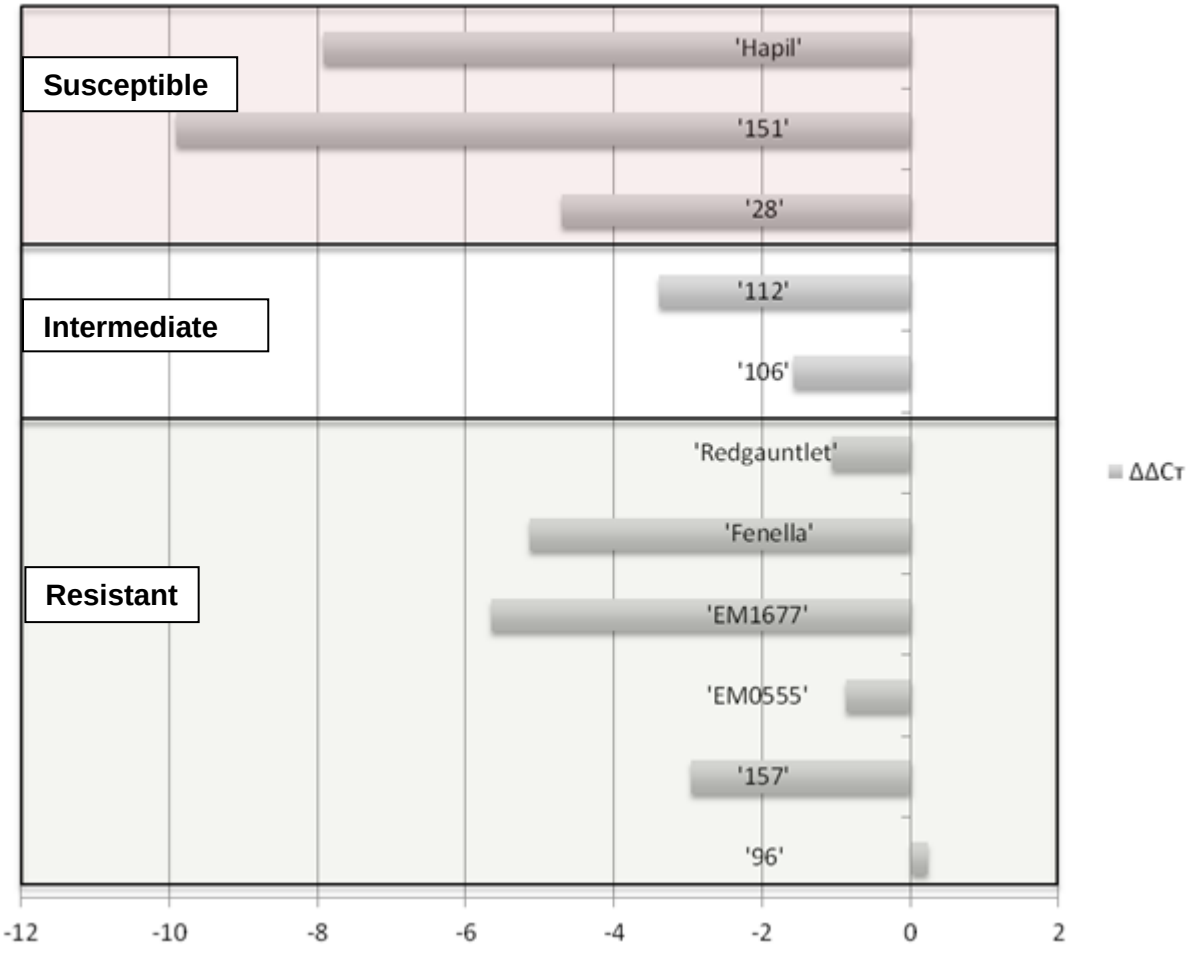
Table 7. Mean wilt susceptibility scores for 46 strawberry lines inoculated with two isolates of *V. dahliae*

	Isolate 12158		Isolate 12253	
Genotype	**Mean wilt score**	**Rating**	**Mean wilt score**	**Rating**
RH004	5.3	I	5.7	I
RH012	1.0	R	*	*
RH014	13.0	S	8.0	I
RH024	4.3	I	1.0	R
RH025	0.5	R	18.0	S
RH026	12.0	S	29.0	S
RH028	1.7	R	25.0	S
RH029	24.0	S	0.0	R
RH030	0.3	R	9.3	S
RH031	10.3	S	17.0	S
RH032	0.0	R	21.0	S
RH039	6.0	I	10.0	S
RH045	4.0	I	10.5	S
RH050	0.7	R	2.5	R
RH054	3.7	I	0.0	R
RH061	0.7	R	24.0	S
RH063	7.7	I	*	*
RH064	0.7	R	5.0	I
RH069	0.0	R	10.0	S
RH071	0.0	R	13.5	S
RH091	13.0	S	13.5	S
RH093	1.5	R	*	*
RH095	0.3	R	12.3	S
RH096	3.5	R	1.0	R
RH106	8.7	S	4.7	I
RH110	0.0	R	11.0	S
RH111	0.5	R	13.0	S
RH112	2.3	R	3.7	I
RH115	0.0	R	27.0	S
RH119	5.0	I	1.0	R
RH122	1.7	R	14.7	S
RH150	*	*	9.0	S
RH151	4.5	I	26.3	S
RH157	2.5	R	0.7	R
RH161	0.0	R	14.7	S
RH164	*	*	*	*
RH179	2.0	R	13.7	S
Cam Favourite	0.3	R	6.0	I
EM0555	0.0	R	4.3	I
EM1677	0.0	R	13.5	S
Fenella	3.0	R	8.7	S
Flamenco	*	*	*	*
Hapil	1.7	R	6.0	I
Merton Dawn	17.0	S	7.5	I
Redgauntlet	0.0	R	2.0	R
Sierra	0.3	R	5.7	I

Quantitative real-time PCR to determine levels of pathogen in plant tissue

Due to the low level of symptom expression in the Year 1 glasshouse experiment it was clear that qPCR analysis of the petioles collected at the conclusion of the experiment would not be informative. It was decided to focus on the plants that had been inoculated with the more pathogenic isolate (12253) in Year 2 and examine the petioles taken from plants from the three resistance categories among the RG x H seedlings, along with the parent cultivars and three unrelated genotypes that all show strong field resistance to wilt.

Figure 1. ΔΔCt (the relative change in the ratio of *V. dahliae* to *F* x *ananassa* DNA between T0 and T1 for plants inoculated with isolate 12253)



Data

Genotype	ΔΔCT	Status
RH096	0.226477729	R
RH157	2.958217727	R
EM0555	0.865188705	R
EM1677	5.667474959	R
Fenella	5.144042333	R
Redgauntlet	1.056795756	R
RH106	1.575857798	I
RH112	3.402218501	I
RH028	4.701176325	S
RH151	9.910872777	S
Hapil	7.929242134	S

Figure 1 shows that, on average, there was quantitatively less *V. dahliae* DNA present in petioles of the resistant lines than in the susceptibles, but this difference was borderline for statistical significance (p=0.056). However, the relationship was not straightforward. For example, EM1677 and EM0555 show a similar level of strong field resistance to wilt but there was a six-fold difference in the quantity of *V. dahliae* DNA detected by the qPCR. Seedling RH096, from the mapping population, appeared to be uninfected with *V. dahliae*.

Discussion

The original results for validation of the marker $HRGP_{232}$, that were reported at the end of Year 1, had been encouraging as this allele was present in 65% of the resistant lines in the validation set and only one among the 18 susceptibles. It was also found to be common in the wider germplasm set, which included modern and old cultivars and breeding lines from a range of origins. However, further analysis of the QTL data from the earlier BBSRC project has now shown that it is allele $HRGP_{213}$ that is most closely linked to the resistance QTL, which makes the results for $HRGP_{232}$ difficult to interpret. It may still be a useful marker for breeding but currently its relationship to resistance remains empirical. $HRGP_{213}$ should be a better marker but it is rare in modern cultivars. However, it was more abundant in the EMSBC breeding lines, some of which have been selected for their field resistance to wilt, and is likely to be most useful in crossing schemes designed to introduce resistance from older germplasm.

The other three markers that were investigated showed a less clear relationship with observed field resistance, as all three were found to be present in some susceptible genotypes, albeit at a much lower frequency than in the intermediate and resistant lines. Two of these markers, RVd6 and RVd7, were found to be common but RVd4 was relatively rare in the modern cultivars and SBC lines and more common in the older germplasm from

the collection. It is clear that all of these markers are potentially useful when breeding for resistance to wilt but they will need to be used in combination, as no single marker is likely to be sufficiently reliable in predicting field resistance.

The relative importance of the different resistance QTL in the wider germplasm set is still unclear at this stage but most of the lines known to have strong field resistance had multiple markers present. For example, Flamenco, EM0555 and EM1624 all had three markers (in different combinations) and these lines have consistently shown strong resistance in field tests. There were a small number of very anomalous results, most notably EM1677 which had no markers but has shown strong resistance in field tests. Also Senga Sengana, which has shown strong resistance to wilt in many northern European countries, but was found to have only the two most common markers. The results for Malling Centenary were also surprising, as it had three markers but field tests at EMR had shown it to be susceptible to wilt. However, anecdotal evidence from large scale trial plantings of Malling Centenary on commercial farms has suggested that it is not particularly susceptible to wilt.

The results from these tests clearly show that resistance to *V. dahliae* is a complex trait that is likely to be under the control of multiple QTLs having a cumulative effect. It is currently not possible to identify the best marker combinations but this will become clearer as the five markers are used in the breeding programme. It is also likely that further resistance QTL will be identified in the future to explain the strong resistance in EM1677 and Senga Sengana. The two HRGP markers were used in the design of the SBC crossing programme for 2013 and the other three markers will be employed for the first time in 2014. Crosses will be designed to increase the likelihood of producing seedlings with multiple markers and promising individuals in the seedlings populations will be genotyped for the five markers from 2014 onwards.

The results from the glasshouse experiment in Year 1 were inconclusive, as the large proportion of plants with no symptoms meant that the data could not be analysed statistically. The Year 2 experiment was more successful but the results were still difficult to interpret due to the large number of plants that died at an early stage. Nevertheless, there was a clear and large difference in pathogenicity between the two isolates, which was surprising, as both had originally been isolated from plants showing clear symptoms of Verticillium wilt. Isolate 12158 had been isolated from the cultivar Everest and 12253 from Elsanta. These two cultivars are unrelated, so there is a suggestion that some isolates of wilt may have the ability to overcome resistances present in certain cultivars but not others, i.e. they show a differential response. This is supported by the significant genotype x isolate

interaction and the fact that some genotypes were more susceptible to the less pathogenic isolate 12158 and showed resistance to 12253, e.g. RH029 and Merton Dawn. This hypothesis will require further investigation.

A major objective of the qPCR analysis was to attempt to distinguish between resistance and tolerance to *V. dahliae*. It had been previously observed in the EMR breeding programme that a high proportion of genotypes showing field resistance to wilt also had strong vegetative vigour (e.g. Florence) but there were exceptions to this (e.g. Redgauntlet and Finesse). The hypothesis was that the very vigorous genotypes were able to tolerate the presence of the *V.dahliae* pathogen whereas the less vigorous types were able to avoid infection. The results from the qPCR do not support this hypothesis, as pathogen DNA was found to be present in all the lines tested except for RH096. The results strongly suggest that there is no clear distinction between resistance and tolerance but that we are dealing with a quantitative resistance controlled by multiple genes which can restrict the development of the pathogen but will very rarely prevent infection completely. The mechanism of the resistance is not clear and will require further investigation.

Conclusions

- Five resistance markers have been validated and all will be useful when breeding for resistance to Verticillium wilt
- Cultivars with the strongest field resistance to Verticillium wilt typically have multiple resistance markers but there are a small number of exceptions to this
- Quantifying the pathogen DNA in infected plants of a range of genotypes did not show that they could be separately classified as either tolerant or resistant
- Resistance is a quantitative trait that is likely to be under the control of many genes having a cumulative affect

Knowledge and Technology Transfer

Results from the genotyping of the proprietary cultivars were provided to Edward Vinson Ltd and Driscoll's Genetics Ltd.

Results from the marker validation work were presented at the HDC/EMRA Soft Fruit Day at East Malling Research in October 2013

Glossary

ABI Genotyping Platform – DNA analysis equipment that will detect presence or absence of specific sequences of DNA. In this case those associated with resistance genes

Allele – An alternative form of a gene (one member of a pair) that is located at a specific position on a specific chromosome

BBSRC – Biotechnology and Biological Sciences Research Council

Conidia – Asexual, non-motile spores of a fungus, in this case *Verticillium dahliae*

Fluorescently labelled primer – a specific DNA sequence used for gene detection by the ABI Genotyping Platform

Germplasm – Genetic resources. In this case a collection of plants of different strawberry cultivars and breeding lines

HRGP – Hydroxyproline-rich glycoprotein. A protein known to be involved in plant defence mechanisms

QTL – Quantitative trait locus, a gene influencing a characteristic that shows a continuous distribution (e.g. plant height) as opposed to discrete differences (e.g. red or white flowers)

qPCR – Quantitative real-time polymerase chain reaction. A method to quantify the DNA of a target gene in relation to an endogenous reference gene

References

Amil-Ruiz, F. et al (2013). Identification and validation of reference genes for transcript normalization in strawberry (*Fragaria* x *ananassa*) defence responses. www.plosone.org 8 (8).

Atallah, Z. K. et al. (2007). Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to Verticillium wilt. Phytopathology 97 (7): 865-872