Project title:	Genetics of resistance to Verticillium wilt in strawberry
Project number:	SF135
Project leader:	Dr David Simpson, East Malling Research
Report:	Annual, March 2013
Previous report:	None
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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

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[The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.]

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.

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GROWER SUMMARY

Headline

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

Background

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 is to validate markers for these resistance genes by looking for their presence or absence in a wide range of unrelated strawberry varieties and correlating this with the field resistance of those varieties to *Verticillium dahliae*. A second aim is to investigate the genetic differences between resistance and tolerance to *V. dahliae*. 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 varieties with strong and durable resistance to wilt.

Summary

In an earlier project, a gene coding for hydroxyproline-rich glycoprotein (HRGP) had been found to be associated with resistance to Verticillium wilt in a progeny of 188 seedlings from the cross Redgauntlet x Hapil. HGRP proteins are known to be associated with plant defence. We tested for the presence of the HGRP gene in 46 varieties 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. The HGRP marker gene was found to have six alternative forms (alleles) of which three were associated with resistance in the 46 lines that formed the validation set. One of these alleles (232) was common and was present in 62% of the resistant lines. Following the validation of the marker, a further 122 strawberry lines were tested for presence of the resistance marker alleles. These lines included modern varieties, old varieties and breeding lines from the EMR collection, and selections and parental lines being evaluated by the East Malling Strawberry Breeding Club (EMSBC). In this wider collection allele 232 was again the most common but it was noticeable that the varieties with strongest field resistance to wilt typically had two different resistance alleles present.

The HGRP marker has been used for the first time in designing the 2013 crossing programme for the EMSBC. All parental lines were screened for presence of the marker so that crosses could be designed that will increase the probability of resistant individuals being present in the seedling population that will be evaluated in 2014.

Financial Benefits

For this interim report it is not appropriate to undertake a cost/benefit analysis

Action Points

There are no action points for growers at this stage of the project

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 many genes contributing to the expression in an additive way, these are known as QTL. This objective was successful and we now have molecular markers for three significant resistance QTL. This intention of this project is 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 varieties and breeding lines, not just those related to Redgauntlet.

Another objective of this project is to develop a molecular method to distinguish between resistance and tolerance to *V. dahliae* by measuring the amount of pathogen DNA in the roots of infected plants. Several varieties that are classified as resistant are very vigorous when grown in the absence of the pathogen, and it may be 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 is to study a subset of the mapping progeny, and quantify the pathogen DNA present in a range of plants showing different symptom expression. If clear differences are found then this will allow us to classify the existing QTL for resistance or tolerance and may also identify additional QTL. It would be desirable to combine both resistance and tolerance in the same variety, as these plants are likely to be very robust when *V. dahliae* is present in the soil.

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 96 EMSBC selections, breeding lines and parental cultivars, 24 modern commercial cultivars (including six proprietary lines from Driscoll's Genetics Ltd and three from Edward Vinson Ltd) and 48 accessions from the EMR collection (168 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 resistance markers in the DNA samples from the 168 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

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 varieties, one mixed isolate and a control with no pathogen.

Plants were observed at weekly intervals and any symptoms of wilting were recorded. After nine weeks, petiole tissue was harvested from all plants and snap frozen in liquid nitrogen.

Results

Marker validation

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.

Initially 46 varieties and breeding lines were genotyped for HRGP. These 46 lines were chosen as a validation set as all had previously been characterised for resistance to wilt on multiple occasions, using a highly infested field plot maintained at EMR. 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. In this validation set, one further allele (237) was identified in addition to the five from RGxH (Table 1).

Allele 232 was present in 13 from 21 (62%) of the resistant lines but in only one from 17 of the susceptibles, while three of the eight intermediates had allele 232. The second allele associated with resistance (213) was less common in these lines but was absent from all the susceptible lines and present in four of the 21 resistant lines (19%). The additional allele (237), which had not been present in the original mapping progeny (RGxH), was fairly uncommon, being present in 17% of the lines but was weakly associated with resistance, being over-represented in the resistant and intermediate lines compared to the susceptibles.

Table 1. Presence (√) or absence of six alleles for HRGP in 46 varieties and breeding lines

	Allele						
Variety	**A (213)***	**B (221)**	**C (224)**	**D (229)**	**E (232)***	**G (237)***	**Class†**
Elsanta		√	√	√			Sus
EM1442		√	√	√			Sus
EM1580		√	√	√			Sus
EM1733		√	√	√			Sus
EM1764		√	√	√	√		Sus
EM1772		√	√	√			Sus
EM1812		√	√	√			Sus
EM1856		√	√	√			Sus
EM1871		√	√	√			Sus
EM1942			√				Sus
EM1966			√				Sus
Emily			√			√	Sus
Eros		√	√	√		√	Sus
Hapil		√	√	√			Sus
Holiday		√	√				Sus
Mae		√	√	√			Sus
Vibrant		√	√	√			Sus
Cambridge Favourite	√	√					Int
Elegance		√	√		√		Int
EM1399		√	√				Int
EM1500		√				√	Int
EM1727							Int
EM1792	√	√		√	√	√	Int
Malling Pearl		√			√		Int
Symphony			√	√			Int
Albion		√			√	√	Res
Alice		√			√		Res
Cupid		√	√	√			Res
EM0555	√	√	√		√		Res
EM0701		√	√	√			Res
EM0972		√	√	√			Res
EM1624	√	√	√		√		Res
EM1677		√	√	√			Res
EM1682			√	√			Res
EM1785		√	√	√	√	√	Res
Everest		√			√	√	Res
Fenella		√	√	√			Res
Finesse		√			√		Res
Flamenco		√	√	√	√		Res
Florence			√		√		Res
Judibell	√	√	√				Res
Pegasus		√	√	√	√		Res
Redgauntlet	√	√			√		Res
Senga Sengana		√			√	√	Res
Sonata		√			√		Res
Viktoriana		√	√	√			Res

*Shaded columns indicate alleles associated with resistance to wilt.

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

Screening varieties, selections and breeding lines for presence of the HRGP marker

Following the initial validation of the marker in the 46 lines, a further 122 lines were screened to determine which had any of the resistance-associated alleles present. The results for all 168 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 EMSBC

Table 2. Presence of three HRGP alleles associated with resistance to *V. dahliae* in 24 modern strawberry cultivars

Resistance allele			
232	**213**	**237**	**None**
Driscoll 2	Amelia	Driscoll 1	Cupid
Driscoll 3	Judibell	Driscoll 2	Driscoll 5
Driscoll 4		Eve's Delight	Camarillo
Elegance		Sweet Eve	Elsanta
Elianny		Verity	Fenella
Evie 2			Flair
Finesse			Rumba
Sonata			Sweetheart
			Symphony
			Vibrant

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

Resistance allele			
232	**213**	**237**	**None**
Aberdeen	Addie	Allstar	Darselect
Addie	Allstar	EM0881	Delmarvel
Alice	Cambridge Fav'rite	EM1500	EM0701
EM0555	EM0555	Emily	EM0791
EMR314	*F. chiloensis* Man Alt	EMR314	EM0972
Korona	Korona	Eros	EM1108
Little Scarlet	Osmanli	*F. moschata*	EM1399
Mara des Bois	Pandora	*F. vesca* Hawaii 4	EM1442
Mimek	Redgauntlet	*F. virginiana* Fre9	*F. chiloensis* BSP14
Nyoho	Royal Sovereign	Little Scarlet	*F. virginiana* III
Osmanli	Sierra	Pandora	Hapil
Pegasus		Providence	Holiday
Perle de Prague		Senga Sengana	Mae
Providence		Sierra	Merton Dawn
Redgauntlet			Perfection
Royal Sovereign			Rainier
Senga Sengana			Siletz
Sierra			Wiltguard
Tribute			

Table 4. Presence of three HRGP alleles associated with resistance to *V. dahliae* in 96 selections, breeding lines and cultivars being used as parents by the EMSBC.

Resistance allele			
232	**213**	**237**	**None**
Alba	Argentera	Albion	DNBL205
Albion	Christine	Clery	EM1580
Argentera	EM1620	Diamante	EM1607
Charlotte	EM1624	DNBL212	EM1677
Clery	EM1786	EM1756	EM1682
Diamante	EM1792	EM1785	EM1727
DNBL212	EM1905	EM1792	EM1733
EM1620	EM1949	EM1795	EM1746
EM1624	EM2053	EMR348	EM1752
EM1704	EM2087	EMR477	EM1772
EM1764	EM2090	EMR537	EM1775
EM1785	EMR474	Everest	EM1781
EM1792	EMR485		EM1812
EM1795	EMR489		EM1837
EM1832	ITA 91-136-1		EM1839
EM1860	ITA 94-153-51		EM1856
EM2034	P82		EM1871
EM2056	P85		EM1888
EM2062			EM1942
EM2064			EM1966
EM2065			EM1977
EM2066			EM1998
EM2090			EM2085
EM2092			EM2131
EM2157			EM2134
EM2170			EM2135
EMR348			EM2150
EMR428			EM2156
EMR437			EM2181
EMR466			EM2182
EMR474			EMR349
EMR485			EMR470
EMR489			EMR556
EMR521			EMR568
EMR537			EMR583
EMR562			Granda
EMR565			SDBL120
EMR569			SDBL123
Everest			SDBL126
Flamenco			
Florence			
Florina			
ITA 91-136-1			
ITA 94-153-51			
Malling Pearl			
Marmolada			
SDBL122			

Allele 232, which was the one most strongly associated with resistance in the validation set (Table 1), was the most common of the three alleles in all three germplasm categories. It was present in one third of the modern cultivars but allele 213 was relatively under-represented in this category. Allele 237 was found in five modern cultivars, all of which were from the two UK proprietary breeding programmes. Some 42% of the modern cultivars had none of the resistance alleles and only Driscoll 2 had two alleles present (232 and 237). Driscoll 2 is a numbered selection that has not yet been released.

In the accession from the EMR collection multiple alleles were more common. Of the 19 accessions with allele 232, 11 also had either 213 or 237 and one cultivar (Sierra) had all three alleles. In the SBC collection, 16 from 45 accessions with allele 232 also had one of the other alleles and one advanced selection (EM1792) had all three alleles.

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 this experiment was very low. Of 288 plants inoculated, only 61 (21%) developed visible symptoms of wilt (Table 5) 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 16h 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.

It will be necessary to carry out a second experiment in year 2 but this will take place earlier in the year, with inoculations planned for late July or early August.

Table 5. 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

Discussion

The results for validation of the marker for the HRGP gene were very encouraging. Allele 232 was present in 62% of the resistant lines tested and in only one line among the 17 susceptibles. This allele was also found to be common when screened in the wider germplasm set, which included modern and old varieties from a range of origins and lines currently under evaluation or being used as parents in the EMSBC. The other alleles associated with resistance (213 and 237) were less common, particularly among the modern varieties, but it was notable that the varieties and breeding lines with the strongest field resistance typically had two of the resistance alleles present. For example, Redgauntlet and EM555 have 213 and 232, Senga Sengana has 232 and 237. Sierra had all three resistance alleles and merits further investigation as a possible parent for breeding. It is a very old Californian variety that would not be adapted in the UK but is reputed to be highly

resistant to North American strains of *V. dahliae*. The HRGP marker is being used in the programme of the EMSBC for the first time in 2013, with crosses being designed based on the presence of the three alleles. This will increase the probability of resistant lines being present in the seedling population to be evaluated in 2014. In future years the marker data will be used to select some additional parents from the EMR germplasm collection, particularly those with more than one resistance allele.

The results from the glasshouse experiment were inconclusive. Although there was an indication of differences in virulence between the *V. dahliae* isolates, the large proportion of plants with no symptoms meant that the data could not be analysed statistically. It will now be necessary to do additional glasshouse experiments in year 2 to investigate genetic differences between resistance and tolerance.

Work in year 2 will also focus on validating markers for the other resistance QTL that were identified in project BB/E007074/1. The encouraging results for HRGP suggest that the other QTLs may also be present in a wide range of unrelated germplasm, opening up the possibility of combining multiple resistance genes in future strawberry varieties.

Conclusions

- Allele 232 of the HRGP marker is widely distributed in a range of unrelated cultivars and breeding lines
- Varieties with the strongest field resistance to Verticillium wilt typically have two resistance alleles for HRGP

Knowledge and Technology Transfer

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

Knowledge transfer activities are planned for year 2 of the project, when more results will be available

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 varieties 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)

