Identification of Black Currant (*Ribes nigrum* L.) Plants with Gall Mite (*Cecidophyopsis ribis*) Resistance Using a PCR Marker Linked to the *Ce* Gene

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Abstract

Black currants can be infested with the arachnid gall mite (*Cecidophyopsis ribis*). Besides reducing yield, *C. ribis* mite is the vector of the Black currant Reversion Virus (BRV), which makes the plant sterile. Because of new restrictions of pesticides in EU, other sources of efficient gall mite protection are currently investigated, and one of these is the *Ce* gene, which originates from gooseberries and provides gall mite resistance. The purpose of this study was to optimize a PCR protocol and identify black currant plants with the resistance gene using a PCR-based marker linked to the *Ce* gene. The optimization lead to a slightly lower annealing temperature and an increased number of PCR cycles for DNA amplification. The gall mite resistance primers and the control primers used appeared to be sensitive, and more dependent on water quality than other primers. A total of 50 plants, of which 12 were cultivars and 38 plants came from the two crossing populations Rib0716 and Rib0712 were analyzed using the optimized protocol. All parental plants of the two populations proved to carry the *Ce* gene marker and should be resistant to *C. ribis*. Since some plants in both populations did not have the *Ce* gene marker, it was established that all parents were heterozygous for the gene. 17 of 30 (55%) in the Rib0716 population and 5 of 7 (71%) in the Rib0712 population was resistant to *C. ribis,* according to the presence of the marker. In case of population Rib0716 the results significantly deviated from the predicted segregation ratio (75% resistant plants). This could be due to a lower viability among homozygous resistant seedlings, resulting in only heterozygous resistant and homozygous susceptible plants, which lowers the theoretical resistance frequency to 67%. This hypothesis, got statistical support and is a plausible explanation of the results from the study.

Introduction

Black currant (*Ribes nigrum* L.) is a crop of commercial interest because of its flavour and colour (Brennan and Graham 2009), but the crop is also of interest due to nutritionally important oils from seeds (Ruiz del Castillo et al 2004), it's high content of phenolic compounds (Ovaskainen 2008) and vitamin C (Benvenuti et al. 2004). Polyphenolic compounds have antioxidant and anti-inflammatory effects (Talbart 2011), which are believed to provide a defence against some diseases, such as cardiovascular and Alzheimer diseases, and some types of cancer (Karjalainen et al. 2009). Vitamin C provides protection against common colds, as well as the previously mentioned cardiovascular diseases and some cancers (Schlueter and Johnston 2011).

The arachnid gall mite (*Cecidophyopsis ribis*) is one of several black currant pests, and causes damages on afflicted plants. Infested plants grow malformed and undergo a reduction in yield (Mitchell 2011). *C. ribis* is also the natural vector of Black currant reversion virus, BRV (Jones and McGavin 2002). The virus causes the black currant to develop fewer and smaller, malformed leafs, and will eventually make the plant sterile (Jones 2000). Efficient gall mite pesticides have recently been restricted in EU for health and environmental reasons (Gordon 2008). It is therefore of interest to breed cultivars of black currant in regard not only to fruit quality, high concentrations of phenolic compounds and vitamin C, but also to natural resistance against diseases and pests like *C. ribis* (Hummer and Barney 2002). In black currants the *P* gene, provides some protection, but the plant can still be infested by *C. ribis* allowing the BRV to be transferred (Andersson 1971). However a crossing between gooseberries and black currants have resulted in black currant cultivars with *C. ribis* resistance from the *Ce* gene, which gives the black currant plant full protection from infestation of *C. ribis* and BRV (Mitchell 2011).

Identifying resistant plants by exposing them to *C. ribis* is time consuming and can give inaccurate results, according to Brennan et al. (2009). To speed up and ease the selection process in regard to *C. ribis* resistance the same authors developed a PCR-based marker linked to the *Ce* gene. The method amplifies 130 base pairs in the *Ce* gene, which makes it possible to distinguish *C. ribis* resistant plants from susceptible with high accuracy.

The purpose of this study was to optimize the available PCR protocol to investigate if the *Ce* gene marker was present in any other than the two known resistant cultivars among 12 black currant cultivars included in the study (see table 1). Further were plants from two different crossing populations; Rib0716 and Rib0712, analyzed for the *C. ribis Ce* gene resistance marker.

Materials and methods

The plants analyzed (Table 2) were 12 cultivars, and 38 plants from two cross-breed populations; 31 plants from the Rib0716 population and 7 of the Rib0712 population. The Rib0716 population is a crossing between the cultivars SK8944-13 and Bri8825-2, where

SK8944-13 is known to have the *Ce* gene from the study by Brennan et al. (2009). The Rib0712 population, a crossing between SK8872-1 and BRi8825-2, has a known resistant parent in SK8872-1 (Brennan et. al. 2008) (Figure 1).

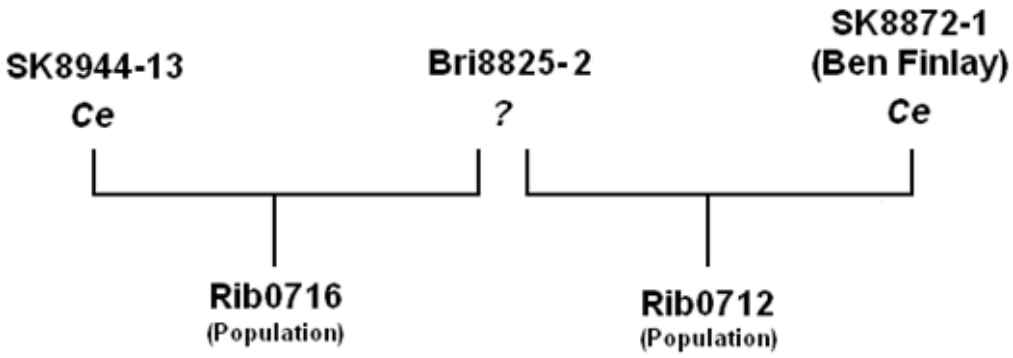

Figure 1: Pedigree of the relationship between the populations Rib0716 and Rib0712. Both populations share one parent, namely Bri8825-2.

Extraction

DNA was isolated from approximately 100 mg fresh or frozen black currant leaves collected in June using the Qiagen Dneasy Plant Mini Kit. The extraction was made according to the manufacturer's protocol, with the exception of the following modifications; in step 1 and 2 the plant material was disrupted in 200 µl Buffer AP1 before adding the remaining 200 µl of the Buffer AP1 and the RNase A. In step 3 the lysate was centrifuged for 10 minutes at 14000 rpm. In step 6 the second time in the centrifuge lasted for 2 minutes. After step 8 the empty column was centrifuged additionally 2 minutes at 14000 rpm to dry the membrane. In the final step 50 µl Elution Buffer was added two times instead of 100 µl at once, and the second turn with the centrifuge lasted for 2 minutes.

PCR

The PCR-trials were executed based on a protocol made by the developers of the specific Gall Mite Resistance primer used in the experiment at the James Hutton Institute in Scotland. The PCR-reactions in the original protocol were performed in 10 µl volume, with the following concentrations: 200 µM dNTPs, 1 µM each of the resistance primers GMRes a L (5' TTA CCG CAG ATA CAA GGT GAA G 3') and GMRes a R (5' GGA CTA GGC CCT CCT ATG AC 3'), 0,5 µM each of the control primers ERB0102N10 L (5' ACA ACT GCC ATC TGT GCA AC 3') and ERB0102N10 R (5' TGT CTT CTT AAC ARG CGG AAA 3'), DNA with the approximate concentration of 1 ng/µl and 0,5 U Taq enzyme per sample of Roches Taq DNA polymerase 5U/µl. The original PCR program was 94°C 5 min followed by 30 cycles of 94°C for 30 s; 59°C for 30 s; 72°C for 30 s and a final extension of 72 °C for 7 minutes. Following optimaization

the annealing temperature was reduced from 59°C to 58°C and the number of cycles was increased from 30 to 40 cycles. The samples were evaluated on a 1.5% agarose gel.

The Optimization of the Protocol

Trials were made with various combinations and concentrations of the different PCR compounds (table 1). DNA was extracted in two different ways, using Qiagen Dneasy Plant Mini Kit and using the CTAB method, and DNA samples were sent from the James Hutton Institute in Scotland. Two batches of primers were bought, and primer samples were also obtained from the James Hutton Institute. The quality of the DNA was surveyed using RAPD primers and black currant specific SSR primers and by analysis performed by the James Hutton Institute, where also the working statuses of the gall mite primers were confirmed.

Roche, Thermo Scientific and GE healthcare Taq enzymes were tried each with their attached buffer, and magnesium was added in some trials. Three different PCR machines and different versions of the original program and the NEO program (94°C 3 min followed by 30 cycles of 94°C for 45 s; gradient 56-66°C for 45 s; 72°C for 45 s and a final extension of 72 °C for 10 minutes), along with several batches of milli-Q water were tried. The different versions of the original protocol conducted in the optimization process can be found in table 1.

After optimization additional trials were made to investigate if the laboratory conditions and temperature affected the PCR reaction substantially, using the optimized protocol and by handling the PCR mixes carelessly and exposing them to warmer temperatures than during normal preparation.

Results

The Optimization of the Protocol

The experiments were initially unsuccessful and several measures were taken in the pursuit for results; the DNA extracted using the Qiagen Dneasy Plant Mini Kit, DNA extracted with the CTAB-method and DNA-samples from the James Hutton Institute were tried with the two ordered batches of primers and the primer sample sent by the James Hutton Institute without success. However, the DNA gave results with SSR and RAPD primers, and both DNA and primers gave results when run in Scotland by the James Hutton Institute.

None of the three different DNA polymerases tried (Roche, Thermo Scientific and GE healthcare) and their attached buffers worked with the GMR and control primers, but did amplify DNA fragments using other primers. Magnesium was added in different concentrations, and several attempts with different concentrations of all compounds were conducted (Table 1). Nucleotides from two batches were tried without comedown for the *C. ribis* resistance, but with positive result using other primers and in unrelated experiments. Three different PCR machines were tried with different times of the PCR steps, temperatures and number of cycles. Three different persons conducted the experiments separately, and

preparation of PCR-mixes and running the PCR-machine was done during all hours of the day to test any effects of the climate in the laboratory caused by the varying weather conditions of a Swedish summer, concerning light, temperature and air humidity.

Using a second batch of water, the experiments resulted in faint bands only visible on pictures taken of the agarose gel (Figure 2). After changing the filter in the water machine the experiments gave clear results, using the lower annealing temperature of 58°C and 40 PCR cycles (Figure 3). Since the first batch of water used seems to have been disposed of, it was not possible to prove the theory that the water caused the problems. Two trials were made with the new water but with the same of the remaining components as when the experiments were unsuccessful; one simulating a warm laboratory on a hot summer's day by leaving the PCR test tubes on a warm fan for approximately 20 minutes before insertion in the PCR-machine, and the other not preparing the samples on ice. Both of these experiments amplified the *Ce* gene marker, which suggests that the temperature in the laboratory did not affect the outcome significantly.

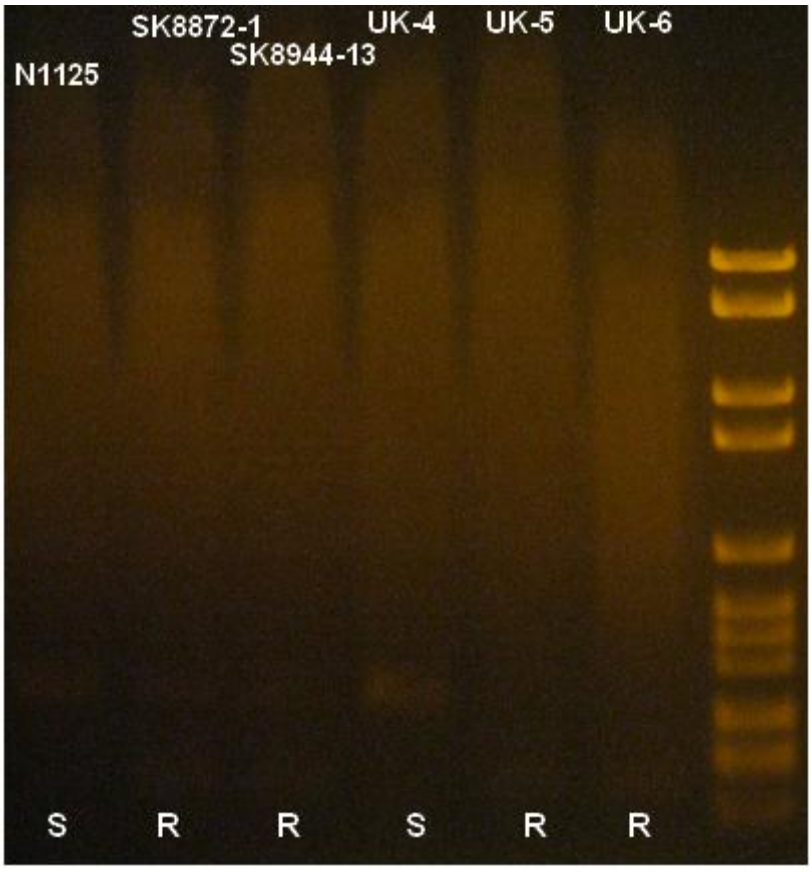

Figure 2: Faint bands, only visible on photography, received after taking water from the second batch. The samples named UK were sent from the James Hutton Institute in Scotland.

The optimized protocol finally used was thus as follows:

> The DNA used was extracted using the Qiagen Dneasy Plant Mini Kit. The PCR-reactions were performed in 10 µl volume, with the following concentrations: 200 µM dNTPs, 1 µM each of the resistance primers GMRes a L (5' TTA CCG CAG ATA CAA GGT GAA G 3') and GMRes a R (5' GGA CTA GGC CCT CCT ATG AC 3'), 0,5 µM each of the control primers ERB0102N10 L (5' ACA ACT GCC ATC TGT GCA AC 3') and ERB0102N10 R (5' TGT CTT CTT AAC ARG CGG AAA 3'), DNA with the approximate concentration of 1 ng/µl and 0,5 U Taq enzyme per sample of Roches Taq DNA polymerase 5U/µl. The PCR program was 94°C 5 min followed by 40 cycles of 94°C for 30 s; 58°C for 30 s; 72°C for 30 s and a final extension of 72 °C for 7 minutes. The samples were evaluated on a 1.5% agarose gel.

Gall Mite Resistance

Results shows that from the plants examined 17/30 (55%) of the Rib0716 population and 5/7 (71%) of the Rib0712 population had the *C. ribis* resistant phenotype based on the presence of the *Ce* gene marker. The results from the experiments showed that BRi8825-2, the common parent in the two analyzed populations, had the marker for the *Ce* gene, which means that both parents of the studied crossing populations have the gene (Table 2 and Figure 3).

Table 2: A list of the examined cultivars and their phenotype based on the presence of the *Ce* gene marker. SK8944-13 and SK8872-1, was used as positive controls since both were known to have the *Ce* gene from previous studies (Brennan et al 2009, Brennan et al 2008).

Cultivar	Combination	Phenotype
Rib0716-01	SK8944-13 x Bri8825-2	R
Rib0716-02	"	R
Rib0716-03	"	S
Rib0716-04	"	S
Rib0716-05	"	R
Rib0716-06	"	S
Rib0716-07	"	R
Rib0716-08	"	S
Rib0716-09	"	R
Rib0716-10	"	R
Rib0716-11	"	R
Rib0716-12	"	R
Rib0716-13	"	R
Rib0716-14	"	R
Rib0716-15	"	S
Rib0716-16	"	S
Rib0716-17	"	R
Rib0716-18	"	S
Rib0716-19	"	R
Rib0716-21	"	R

Rib0716-22	"	S
Rib0716-23	"	S
Rib0716-24	"	S
Rib0716-25	"	S
Rib0716-26	"	R
Rib0716-27	"	S
Rib0716-28	"	R
Rib0716-29	"	R
Rib0716-30	"	S
Rib0716-31	"	x
Rib0716-32	"	R
Rib0712-69	SK8872-1 x Bri8825-2	S
Rib0712-70	"	R
Rib0712-71	"	R
Rib0712-72	"	R
Rib0712-73	"	R
Rib0712-74	"	R
Rib0712-75	"	S
Bri8825-2		R
Bri8919-1		R
Bri8916-5		R
Bri9344-1		S
Bri9504-2-227		S
Bri9508-3A		S
Ben Gairn		S
Poesia		S
Titania		S
N1125		S
SK8872-1 (Ben Finlay)		R (positive control)
SK8944-13		R (positive control)

A statistic analysis was made to evaluate the resistance frequencies based on two hypotheses; one where 75% were resistant, and one where 66.6% were resistant due to lower viability among plants homozygous for the *Ce* gene.

Table 3: Statistic analysis of the resistance frequency received in the study. The Rib0712 population was too small to be analyzed separately, and was therefore analyzed together with the Rib0716 population. The analysis suggests that the 25 susceptible: 75 resisatant hypothesis can be discarded. However, the 33 susceptible:66 resistant hypothesis seems to be a possible explanation of the outcome.

Population	Category	Observed	Proportion	Expected	χ^2	P-value	Hypothesis
Rib0716	Resistant	17	0.75	22.5	5.37778	0.02	Discarded
	Susceptible	13	0.25	7.5			
Rib0716 + Rib0712	Resistant	22	0.75	27.75	4.76577	0.029	Discarded
	Susceptible	15	0.25	9.25			
Rib0716	Resistant	17	0.67	20	1.35	0.2453	Possible
	Susceptible	13	0.33	10			
Rib0716 + Rib0712	Resistant	22	0.67	24.7	0.87	0.3510	Possible
	Susceptible	15	0.33	12.3			

Discussion

The Optimization of the Protocol

In order to optimize the PCR reaction, the annealing temperature was lowered by 1°C and 10 cycled were added. It was indicated that the pureness of the water was of a great importance in order to make the reaction work.

Trial 15-18 in table 1 indicates that changing the water made the experiments work. This is based on the absence of bands in trial 16. In trial 16 the concentration of GMR primers, Taq enzyme, and DNA was the same as in 15, 17 and 18. The absence of control primers was not likely to have affected the amplification of the specific GMR primer negatively. The concentration of dNTPs was the same as in 17 and 18. The numbers of PCR cycles were fewer in 15 and 16 than in 17 and 18, but 15 did provide faint bands despite a very low nucleotide concentration. The major difference in the four trials concerns the batches of water was used. Despite the lower annealing temperature, compared to 15, 17 and 18, of 57°C trial 16 experiments was not successful, and did not result in any bands at all. An additional argument pointing at the primers sensitivity for the water used is the experiment investigating the laboratory temperatures impact on the reaction, which showed that the temperature impact was negligible.

The fact that the experiments started to work after changing the water might be a coincidence or caused by a factor not accounted for here. Not being able to try the old water after succeeding with the experiments using a new batch of water made it impossible to confirm something in water batch 1 interfered with the PCR reaction and obstructed the optimization process. However, because of the number of times the experiment was repeated in different versions of the original protocol any other solution should have been discovered earlier in the optimizing process before the water was changed. That leads to believe that the water is the most plausible explanation of the failed experiments, and points to the importance of clean water when using the gall mite resistance primers.

Gall Mite Resistance

The results show that five cultivars and not only the two expected cultivars among the 12 studied had the marker for the *Ce* gene. Among these the mutual parental of the two crossing populations was found.

The PCR method to identify plant carrying the *Ce* gene is clearly faster and more efficient compared to exposing the plants to gall mite to reveal resistance (Brennan et al 2009). However it does not take other sources of resistance, eg the *P* gene, into consideration. The method could in case of mutations in the analyzed plants provide misleading results; a plant with a mutation in a primers binding site could after analyze appear to be susceptible, and plant with a mutation inhibiting gene activity could appear to be resistant. A possible consequence of the results from an early evaluation of one single gene in a crossing

population is termination of plants lacking the gene in question during the selection process (Mazeikeine I 2012). Early termination in regard to only one gene could lead to a loss of plants with other valuable properties or gene combinations.

In the crossing populations all parents were resistant to *C. ribis*. The *Ce* gene is dominant, which means that the genotypes homozygous resistant and heterozygous resistant both result in a gall mite resistant phenotype. In the left inheritance pattern in Figure 4 one heterozygous resistant and one susceptible parent will result in 50% resistant plants in their offspring. The middle pattern with two heterozygote parents for the *Ce* gene results in the theoretical frequency of 75% resistant plants. The right inheritance pattern, with one heterozygote and one homozygote parental for the *Ce* gene results in 100% resistant plants. Based on the fact that all parents have the resistance gene and that the combinations have lead to susceptible offspring according to the absence of *Ce* marker, it is possible to conclude that all three parents in the two populations are heterozygous.

	A	a
a	**Aa (R)**	**aa (S)**
a	**Aa (R)**	**aa (S)**

50% R

	A	a
A	**AA (R)**	**Aa (R)**
a	**Aa (R)**	**aa (S)**

75% R

	A	a
A	**AA (R)**	**Aa (R)**
A	**AA (R)**	**Aa (R)**

100% R

Figure 4: Phenotypic frequencies of resistant plants (R) depending on parental genotype. (S) means susceptible. A capital A represents the presence of the *Ce* gene, and the lower case a represents absence of the same.

The experiment showed that 71% and 55% of the plants have the marker for the *Ce* gene. In the case of the latter population, Rib0716, the resistance frequency was significantly lower than expected.

An explanation to the obtained values concerning segregation could be contamination during pollination, which can occur despite precautionary measures. Another explanation of why fewer plants than expected had the markeris that black currant plants homozygous for the *Ce* gene may not be viable, and therefore result in a segregation frequency of 2/3. This hypothesis was collaborated by a statistical analysis (table 3),although due to the limited number of tested plants, the results do not necessarily represent the actual resistance frequency of the two populations. Screening of additional plants from larger populations should give more reliable results. It might also be interesting to investigate why the homozygous plants are less viable, if this hypothesis proves to be correct.

The results from this study in combination with further analysis of other plant qualities could be of importance in the quest for black currant cultivars for commercial or scientific interest, and in future breeding programs. The certainty of the results is high, indicated in the study

by Brennan et al (2009), so the phenotype based on results from this study is with a high probability correct.

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Appendix

Table 1: The different versions of PCR mixes used in the trials. The column named **Orig.** lists the concentration of all components of the original protocol. In the row named **Taq Brand** the letter **R** refers to Roche, **GE** to GE Healthcare and **TS** to Theromo Scientific. The number of PCR cycles is 30 if nothing else is specified. The number in the row named **PCR program** retails the annealing temperature. **Grad** means a gradient between the temperatures mentioned. The program **Neo grad** refers to the Neo program described in Materials and Method.

Component	Orig.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
dNTPs (µM)	200	1000	1000	2000	2000	1000	1000	1000	2000	1200	1000	1000	200	200	200	2	200	200	200
MgCl2 (µM)		2000				60		60		1000			2500						
GMRes a L (µM)	1	1	1	1	1,5	1	1	1	1	12	1	1	0,4	1		1	1	1	1
GMRes a R (µM)	1	1	1	1	1,5	1	1	1	1	12	1	1	0,4	1		1	1	1	1
ERB0102N10 L (µM)	0,5	1	1												1	0,5		0,5	0,5
ERB0102N10 R (µM)	0,5	1	1												1	0,5		0,5	0,5
Taq brand	R	GE	R	GE	GE	R	TS	TS	GE	GE	R	R	R	R	R	R	R	R	R
Taq U/µl	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,5	0,04	0,05	0,05	0,04	0,05	0,05	0,05	0,05	0,05	0,05
SDW batch		1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	2	3
DNA	1x	1x	1x	1x	1x	1x	1x	1x	1x	0,8x	2x	2x	0,8x	1x	1x	1x	1x	1x	1x
PCR program	59	57	57	57 & 59	57 & 59	57 & 59	Grad 50-60	Grad 50-60	Grad 50-60	Grad 50-60	Grad 50-60	Grad 50-60	Neo grad	58	58	59	57	58, 40 cycles	58, 40 cycles
Result		X	X	X	X	X	X	X	X	X	X	X	X	X	X	Faint bands	X	Clear bands	Clear bands

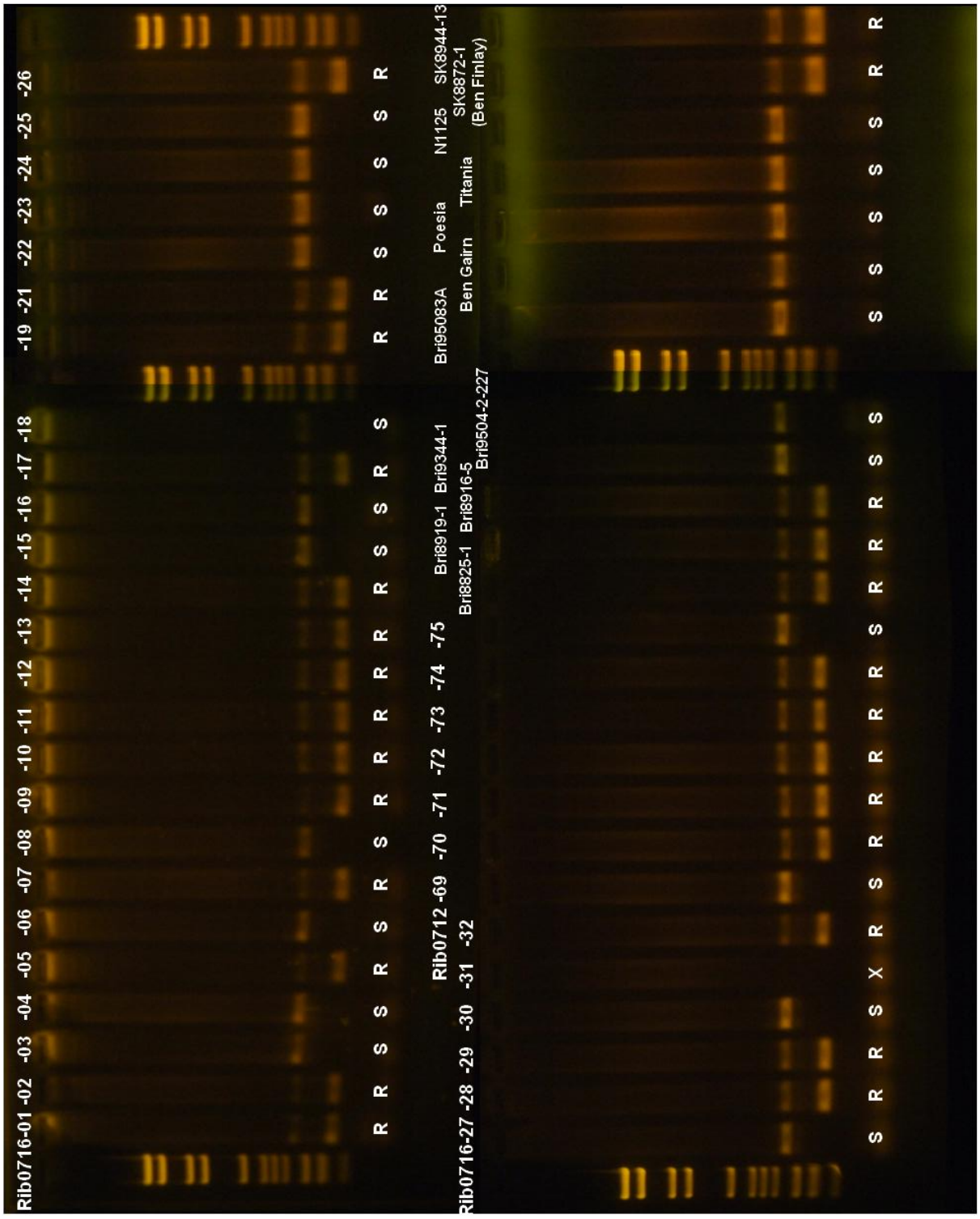

Figure 3: Picture of the gels with the results concerning gall mite resistance. Two bands, or only one visible small band, mean that the plant is resistant to Gall mite, and only one band means susceptible.