

IDENTIFICATION OF NEW RESISTANCE SOURCES FROM DIPLOID WILD STRAWBERRY AGAINST POWDERY MILDEW PATHOGEN

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Powdery mildew pathogen causes diseases in berries and resistance breeding is hampered by the lack of sufficient sources. While control of fungal pathogens relies on chemical fungicides. In either case, a reliable source of resistance for breeding purposes is imperative for efficient protection of the crop plants. A powdery mildew isolate designated GOU1 has been identified as *Podosphaera aphanis* var. *aphanis* using light microscopy and sequencing the ITS region. Pathogenicity tests on 3 diploid wild strawberry (*Fragaria vesca* L.) ecotypes with GOU1 revealed 3 distinctive interaction phenotypes; enhanced susceptible (ecotype 1), intermediate susceptible (ecotype 2) and resistant (ecotype 3). *In vitro* staining methods with diaminobenzidine and trypan blue showed massive mycelial web and conidiophore production on the susceptible ecotype, but there was no mycelia and conidiophore production on the resistant ecotype 3. In the ecotype 3 conidiospore penetration was associated with accumulation of hydrogen peroxide production on the host cells. These findings suggest that these ecotypes could be explored as resistant gene sources to powdery mildew fungus.

Keywords: *Fragaria*, resistance, fungal diseases, pathogen infection, pathogenicity

INTRODUCTION

Today, cultivated strawberries (*Fragaria x ananassa* Duch.) are produced all over the world with more than 4.366.662 tones (t) including 299.940 t strawberry production in Turkey (FAO, 2010). Turkey's strawberry production has been increased at least twice with several disease and agronomic problems over the last decade. Among the strawberry diseases the most destructive strawberry problems come from fungal pathogens.

Powdery mildew of strawberry caused by the obligate parasite *Podosphaera aphanis* (Wallr.) U. Braun & S. Takamatsu var. *aphanis* (syn., *Sphaerotheca macularis* f. sp. *fragariae* (Wall. Ex Fries), is one of the major fungal diseases of this crop worldwide (Spencer, 1978; Maas, 1998, Braun & Takamatsu, 2000). The pathogen affects leaves, petioles, runners, flowers, and fruit, and appears to be specific to this crop (Amsalem *et al.*, 2005; Agrios 1997; Azmat and Khan, 2014). Serious damage to foliage results in reduction of photosynthesis due to dense mycelial coverage, which can lead to necrosis and eventual defoliation (Maas, 1998). Mild infection on fruit causes slight discoloration and shortened shelf life, while more severe infection causes deformation and cracking in fruit. Crops grown in warm, dry Mediterranean climates are particularly vulnerable (Amsalem *et al.*, 2005).

The standard method to control powdery mildew in strawberry nurseries relies on the application of sulfur and systemic fungicides. However, different pathogen populations may differ in their sensitivities to these fungicides. Resistance to benzimidazole, strobilurin and the demethylation-inhibiting (DMI) fungicides have been well-documented mainly in cucumber and grape powdery mildew (Erickson and Wilcox, 1997; McGrath and Shishkoff, 2001; Ishii *et al.*, 2001). Furthermore, the maximum legislated limits for pesticide residues in food have been determined in European countries (Hall, 1995; Sombardier *et al.*, 2010). In response to environmental and health concerns about the widespread use of pesticides, there is considerable interest in finding alternative control strategies for crop diseases in integrated pest management.

There are no strawberry cultivars that are resistance to powdery mildew and other serious diseases and which have acceptable fruit quality and shelf life (Pertot *et al.*, 2008). However, local differences may exist, reflecting differences in usage patterns, genetic differences among pathogen subpopulations or interactions between the pathogen and local plant varieties. Hence, genetically resistant strawberries need to investigate and their durable resistance mechanisms control the fungal diseases.

Wild diploid strawberries (*Fragaria vesca* L.) grow in all woodland areas from the eastern part of Turkey to the western part of Europe and produce much smaller fruits than

commercial strawberries. The fruits ripen from early to late summer depending on altitude. Wild strawberries have 14 chromosomes, which is the lowest chromosome number of all the strawberry species (Hancock and Luby, 1993). Studying wild strawberries has several advantages; they are diploid (Hancock and Luby, 1993), require only a small space to grow, have a short reproductive cycle, grow in nutrient-poor soils, at all altitudes, produce with stolons, have a small genome size and genetic manipulation is possible (Nam *et al.*, 1999). Therefore, the wild diploid strawberries are good model organisms for investigating novel gene sources for resistance to abiotic and biotic stress inducers (Hancock and Luby, 1993). The diploid wild strawberry has a haploid genome size of 164 Mega base pairs (Mbp) while tetraploid and octoploid strawberries have much larger genome sizes (Dirlewanger *et al.*, 2004).

It is worthwhile to study wild strawberry host and obligate powdery mildew pathogen interactions to investigate host-pathogen interactions for investigating novel resistance sources to the powdery mildew diseases. In the last decade several papers addressed powdery mildew-*Arabidopsis* interactions. The powdery mildew-host reactions involved in gene-for-gene interactions where plant disease resistance genes recognize powdery mildew pathogens and subsequently trigger defense responses (Xiao *et al.*, 1997; Adam *et al.*, 1999; Xiao, 2006).

This paper aims to identify new suitable resistance sources in wild strawberry against powdery mildew pathogen for controlling their diseases on cultivated strawberries and other berries. Strawberry powdery mildew was identified with light microscopy and its internal transcribed spacer (ITS) sequences.

MATERIAL AND METHODS

Plant and fungal material: Wild strawberries were collected from 36°30'0" East (E) - 36°39'0" E longitude to 40°45'0" North (N) - 40°56'0" N latitude at 1083-1278 metres (m) altitude. Morphologically different 6 wild strawberry phenotypes were collected from above coordinates. To compare phenotypes of the wild strawberries with cultivated strawberries, camarosa and delmarvel (*Fragaria x ananassa*) were used in pathogenicity tests. In another experiment, 10 cultivated strawberries, honeoye, delmarvel, tudla, aliso, moralina, annapolis, elvira, muir, tufts and tiago were inoculated with GOU1 powdery mildew. The plants were placed in pots containing 1 soil : 1 manure ratio mix and the pots were placed in a cold room for 24 h at 4°C to ensure a constant environment. The plants were then placed in a controlled environment room with 16 hour (h) day 8 h night conditions at a temperature of 22±2°C.

The inocula were collected from naturally infected leaves of wild strawberry ecotype 1 originating from the above geographic locations and designated GOU1 (Gaziosmanpasa

University1) during July to September 2007. The isolate GOU1 was propagated from a single spore and maintained on wild strawberry ecotype 1 in a growth chamber. Conidia were obtained from fresh sporulating lesions whenever required. At 2 week intervals, new strawberry ecotype 1 plants were inoculated with the GOU1 isolate using the methods described below.

Strawberry ecotypes: Collected 6 wild strawberry ecotypes were individually propagated in pots using their stolons and rhizome like structures just beyond their rootstocks. Genomic DNAs were extracted from 6 wild strawberry ecotypes according to Doyle & Doyle (1987) with modified CTAB extraction method. The extracted DNAs were used to detect polymorphism with four Inter-Simple Sequence Repeat (ISSR) primers in PCR reactions according to Cekic *et al.* (2001) for dissection of wild strawberry ecotypes. The four ISSR primer sequences were: 807: 5'-AGAGAGAGAGAGAGAGT-3' (Annealing temperature (A_t: 50°C), 835: 5'-AGAGAGAGAGAGAGAGYGC-3' (A_t: 54°C), 888: 5'-BDBCACACACACACACA-3' (A_t: 52°C), and 889: 5'-DBDACACACACACACAC-3' (A_t: 52 ° C). The amplification products were run on 3% metaphore agarose gel containing 0.35 µg ml⁻¹ ethidium bromide. The amplified bands were pictured and analysed with Vilber Lourmat (France) gel documentation system. The relationship between 6 ecotypes was displayed as a dendrogram constructed using Biocap software based on Unweighted Pair Group Method using Arithmetic averages (UPGMA). Additionally, the collected and propagated 6 wild strawberry ecotypes were assessed with root and flower morphology to distinguish each ecotype. Each ecotype plant was artificially propagated from its rootstocks or stolons. For pathogenicity tests, 20 wild and cultivated strawberry plants from 3 wild ecotypes and 11 cultivated strawberries were used; half of them inoculated with the powdery mildew and the other halves were not inoculated placing them in separate identical conditions. The pathogenicity tests were repeated at least 5 times as described below.

DNA extraction from the fungal pathogen: Powdery mildew mycelia and spore mixture was collected from heavily infected susceptible diploid strawberry ecotype 1 leaves at 15 days post inoculation. Genomic DNA was extracted using commercial DNA extraction kit (Promega Co, USA) according to manufacturer's protocol. DNA was dissolved in 20 µl distilled sterile H₂O.

DNA sequencing: PCR amplification was carried out on genomic DNA of powdery mildew. Single DNA fragment of approximately 950 bp was amplified by PCR using NS7 (5'-GAGGCAATAACAGGTCTGTGATGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers derived from the conserved 18S and 28S ribosomal genes (White *et al.*, 1990). Sequencing reactions were performed by the convenient TermoSequanase™ (Pharmacia) dye terminator

cycle sequencing pre-mix kit and run on an ABI™ fluorescent sequencing instrument.

Inoculation of plants with powdery mildew: Inoculation with powdery mildew was performed using a fine artist's brush to carefully brush conidia from heavily infected leaves onto the target leaves. Brushing of the infected leaves was carried out in an airtight environment at a height of approximately 5-10 cm above the target leaves, in order to achieve an even distribution of conidia on settling. Cultivated and wild strawberry plants were watered prior to inoculation to maintain high humidity in pots because spores of powdery mildew can germinate in water. Preliminarily, the plants infected by powdery mildew were scored visually, except in providing a uniform comparison between susceptible and resistant wild strawberry plants. Density of deposited spores on the leaves was estimated by counting the number of spores in a 1 cm² area under a microscope. Deposition density ranged from 150-200 spores per cm².

Scoring the GOU1 disease reaction phenotype: The infected plants were assessed for disease development at 7, 14 and 21 days post inoculation (dpi). To determine host range on cultivated and wild strawberry plants, a basic numerical system was used to evaluate the disease reactions (DR) phenotype, based on the extent of mycelial development and conidiation (Adam and Somerville, 1996). Five categories were determined; no or very limited hyphal growth with no conidiation (DR: 0), low to moderate hyphal growth with conidiation (DR: 1), abundant mycelial development with moderate conidiation (DR: 2), excessive conidiation with >30% leaf surface covered by powdery mildew (DR: 3), and finally almost all leaf surface covered by powdery mildew (DR: 4). Ten plants of each ecotype were examined. Data were subjected to ANOVA, and mean separation was conducted by the least significant difference (LSD) test ($P \leq 0.05$) with the SAS statistical analysis package (version 8.2).

Epifluorescence microscopy: Whole leaf samples were immersed in fresh aqueous solutions of the fluorescent dye 3,3'-dihexyloxycarbocyanin iodide at 50 $\mu\text{g ml}^{-1}$. DiOC6 prepared from DiOC6 stock solution in ethanol (0.5 mg ml⁻¹, stored at -20°C as described by Duckett and Read, (1991), left for between 1 and 2 minutes. The leaf samples were then placed on slides and viewed under a Nikon phase contrast microscope fitted with an epifluorescence filter B-2A (excitation filter 450-490 nm).

Diaminobenzidine (DAB) and trypan blue staining: The hydrogen peroxide staining method was used for *in vivo* detection of early responses of powdery mildew attack. Inoculated and uninoculated strawberry leaves were excised at the bases of their petioles and placed in 1 mg ml⁻¹ 3,3'-diaminobenzidine-HCl (DAB, Sigma) at pH 3.8. The leaves were then incubated in a growth room for 10 h at 22°C. For trypan blue staining, leaves were placed in boiling ethanol to remove chlorophyll, then placed in 250 $\mu\text{g ml}^{-1}$

trypan blue solution (1 v lactic acid : 1 v glycerol : 1 v water). The leaves were covered with a cover slip for at least 30 min. at room temperature to allow the fungal spores and hyphae to uptake the trypan blue. On observation, the H₂O₂ became reddish brown and the fungal structures were stained blue.

Establishment of F₁ hybrids: Several attempts were made to produce F₁ hybrids among ecotypes. The ecotype 3 flower buds were emasculated and hand-pollinated with pollen from ecotypes 1 and 2. The ecotypes 1 and 2 are female sterile strawberries and do not have ovaries within their flowers and were therefore used as a pollen source rather than as receivers. The pollinated flowers were bagged to prevent wind pollination and to allow the seeds to set.

RESULTS AND DISCUSSION

Characterization of wild strawberry ecotypes: The root and flower morphologies determined that 3 ecotypes phenotypically different among wild strawberries. Wild strawberry ecotype 1 and ecotype 3 had hairy root, however, ecotype 2 had tap root. On the other hand, the ecotype 1 and ecotype 2 were female infertile and ecotype 3 was fertile that produces small strawberry fruits.

The PCR with ISSR primers was suitable for the molecular characterization of wild strawberry ecotypes. The PCR fingerprints obtained with our assays showed substantial genetic diversity among 3 wild strawberry ecotypes. The ISSR amplified bands were scored for the presence (1) or absence (0). The total number of bands, the distribution of bands across all ecotypes, polymorphic bands, species-specific bands and average number bands per primer were calculated. The four ISSR primers were screened with 6 wild strawberry ecotypes, and all primers were found polymorphic and produced clear and reproducible amplification patterns. The average polymorphism with primers; UBC807 was 4.25, UBC835 was 3.43, UBC888 was 7.17 and UBC889 was 5.2 found. The dendrogram based on ISSR data was constructed by UPGMA analysis, grouping all of the studied ecotypes into three major clusters (Fig. 1).

The GOU1 powdery mildew is Podosphaera aphanis var. aphanis: The powdery mildew, GOU1 sexual stage found on its natural host ecotype 1 was identified as a member of *Podosphaera* species based on the morphology of asci. Microscopic observations on infected leaves revealed that the conidia formed chains and were hyaline, ovoid, and measured 22.0 to 35.0 μm high \times 17 to 23.0 μm wide. Fibrosin bodies were present in these conidia. Chasmothecia were numerous, spherical, amber colored, and their diameters ranged from 80.0 to 105.0 μm (average 95.0 μm). Each chasmothecium contained one ascus with eight ascospores. The ascospores measured 25.5 to 27.5 \times 12.5 to 16.5 μm (average 26.0 \times 14.0 μm). On the basis of these

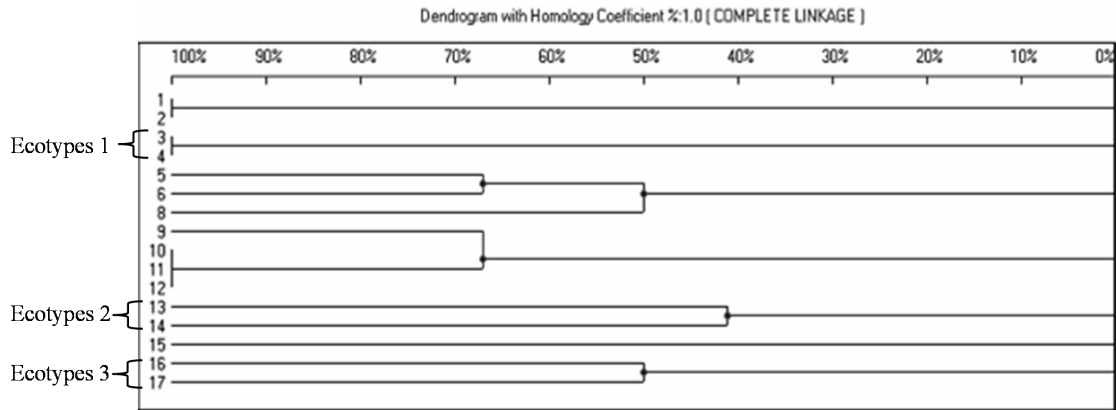


Figure 1. Diversity of wild diploid strawberries. The dendrogram was established based on 4 ISSR data.

characteristic morphologies, the causal agent was determined to be *Podosphaera aphanis* (Wallr.) U. Braun & S. Takamatsu var. *aphanis* (Braun and Takamatsu, 2000).

Sequence verification of powdery mildew DNA: Approximately 950 bp fragment was obtained from polymerase chain reaction (PCR) using NS7 and ITS4 primers. The powdery mildew GOU1 sequence included a portion of the 3' end of the 18S rDNA, the complete 5.8 rDNA subunit and the two flanking ITS1 and ITS2 sequences. As we expected the 3' end of 18S, the 5.8S, and 5' end of 28S rDNA sequences displayed identical sequences with *Podosphaera aphanis* var. *aphanis* sequences. The powdery mildew GOU1 sequence was submitted to National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) GeneBank system with an accession number of HQ918168.

Powdery mildew-strawberry interaction phenotypes: Individually tested 11 strawberry cultivars, honeoye, tudla, aliso, moralina, elvira, muir, tufts, tiago, camarosa and delmarvel (*Fragaria* × *ananassa*) were produced resistant phenotypes with no spore germination (DR: 0) at 14 dpi (Table 1). However, annapolis strawberry cultivar was produced powdery mildew appearance at 14 dpi (Table 1). Inoculated wild diploid strawberries were characterized by the development of a powdery-like fungal growth on the surface of the leaf at 14 dpi. Three disease reaction (DR) phenotypes were distinguished (Table 1) with powdery mildew inoculations (Fig. 2A,B,C): in phenotype 1 strawberry leaves were covered with massive powdery mildews covering both leaf surfaces and plant stems (DR: 4; Fig. 2A), in the second phenotype partial susceptibility was observed on the edge of the adaxial leaf side with massive powdery appearance on the abaxial leaf surface (DR: 2; Fig. 2B), and in the third phenotype, no fungal growth (Table 1) on either leaf surfaces or stems (DR: 0) was observed (Fig. 2C).

Table 1. Pathogenicity test results of *Podosphaera aphanis* var. *aphanis* GOU1 to 3 wild strawberry ecotypes and 11 cultivated ecotypes.

Strawberries	Inoculation ^a	Powdery mildew colonization on leaves (%) ^b	Average disease reaction ^c
Ecotype 1	GOU1	88.6	4.2
	dsH ₂ O	0	0
Ecotype 2	GOU1	42.2	1.9
	dsH ₂ O	0	0
Ecotype 3	GOU1	0	0.1
	dsH ₂ O	0	0
Camarosa	GOU1	0	0.3
	dsH ₂ O	0	0
Delmarvel	GOU1	0	0.2
	dsH ₂ O	0	0
Honeoye	GOU1	0	0.2
	dsH ₂ O	0	0
Tudla	GOU1	0	0.2
	dsH ₂ O	0	0
Aliso	GOU1	0	0.3
	dsH ₂ O	0	0
Moralina	GOU1	0	0.2
	dsH ₂ O	0	0
Elvira	GOU1	0	0.2
	dsH ₂ O	0	0
Muir	GOU1	0	0.1
	dsH ₂ O	0	0
Tufts	GOU1	0	0.2
	dsH ₂ O	0	0
Tiago	GOU1	0	0.3
	dsH ₂ O	0	0
Annapolis	GOU1	74.4	3.6
	dsH ₂ O	0	0

^a 10 plants were used for each inoculation. GOU1: *Podosphaera aphanis* var. *aphanis* isolate, dsH₂O: distilled sterile water (control); ^b Symptoms were evaluated at 14 days post inoculation (dpi); ^c Disease reactions were assessed at 14 dpi

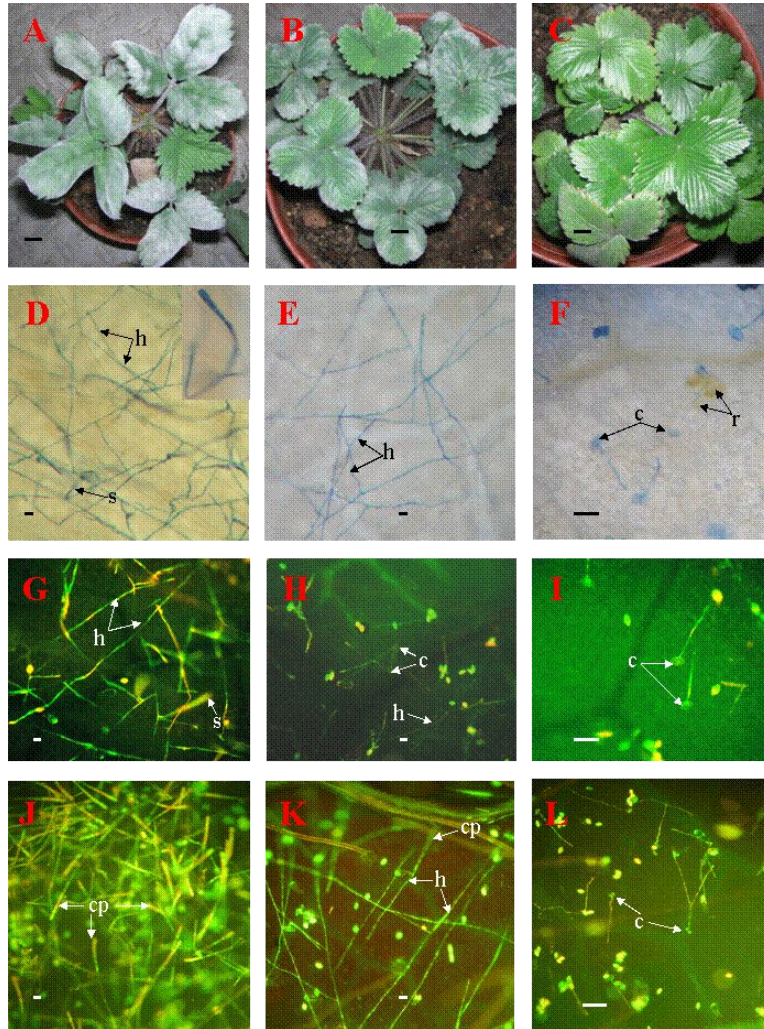


Figure 2. Wild diploid strawberries were inoculated with conidiospores from *Podosphaera aphanis* var. *aphanis* and examined in a time course experiment. A. Ecotype 1 supported growths of massive white mycelium on both sides of leaves, indicating high susceptibility; B. Ecotype 2 showing partial mycelial growth on the edges of the adaxial leaf sides with massive powdery appearances on abaxial leaf surface, indicating intermediate susceptibility; C. No evidence of fungal growth on ecotype 3, indicating resistance. Mycelial growths were shown on three ecotypes from adaxial side at 48-hours post inoculation; D. Ecotype 1 genotype supported massive hyphae production and conidia spore production (inset picture in D); E. a less dense mycelial web was obtained without any conidiospore formation in the ecotype 2 adaxial side at 48-hours post inoculation (hpi); F. accumulation of H₂O₂ in ecotype 3 strawberry cells and presence of conidiospore and germination tube were revealed by 3,3'-diaminobenzidine and trypan blue staining. The H₂O₂ production was visible as a brownish color in the ecotype 3 (F) but was absent in the ecotype 1 and 2 leaf host cells (D, F). The ecotypes were stained with fluorescent dye 3,3'-dihexyloxycarbocyanin iodide and similar results were found in the three ecotypes (G, H, I) at 48 hpi respectively. The enhanced susceptibility of ecotype 1, intermediate susceptibility of ecotype 2 and resistance of ecotype 3 were photographed with the fluorescent dye at 168 hpi (J, K, L) respectively. The A, B and C bars represents 10 mm, in the rest the bars represent 20 μm. h: fungal hyphae, c: conidia, cp: conidiophores, s: conidia sporangia, r: H₂O₂ reaction.

In the first twenty-four hours after spore inoculation with *P. aphanis* var. *aphanis* GOU1, more than 95% of the conidial inoculums were germinated and produced short germ tubes terminating in appressoria on ecotype 1 and 2 strawberry leaves. However, only 20% of the conidial inocula were

germinated and produced germ tubes on ecotype 2 strawberry leaves (data not shown). Within 96 hours of spore inoculation, conidiophores and conidia formation were detected with extensive branched mycelial networks of more than 50 mm in length on ecotype 1 leaves (Fig. 2D and inset

picture). Similar but less extensively branched mycelial network was found without conidiophores and conidia formation on ecotype 2 leaves (Fig. 2E). On ecotype 3 conidia spores produced long germ tubes terminating in appressoria. When an appressorium penetrated host cells, the reaction was associated with accumulation of hydrogen peroxide in ecotype 3 cells and the presence of conidium germ tubes was observed by 3,3'-diaminobenzidine and trypan blue staining (Fig. 2F). These results were confirmed with fluorescent dye 3,3'-dihexyloxycarbocyanin iodide staining at 96 hour post inoculation (Fig. 2G, H, I).

By 168 hours post inoculation (hpi), wild strawberry ecotype 1 leaf surfaces produced massive conidiophores, conidia and mycelial web development detected with the fluorescent dye (Fig. 2J). Less extensive mycelial web with few conidiophores was detected on ecotype 2 leaf surfaces (Fig. 2K). Neither conidiophore formation nor mycelial web development was found on ecotype 3 leaves, and 5% of conidiospores were not germinated at 168 hpi (Fig. 2L). These *in vitro* staining studies clearly identified two distinctive reaction phenotypes: the first susceptible phenotype with enhanced conidiophores, and mycelial web formation with no detectable H₂O₂.

In the second resistant phenotype spore germination ended with penetration without further growth; the spore penetration was associated with H₂O₂ production in the host cells (Fig. 2F).

Strawberry crosses: Many crosses were conducted between susceptible and resistant genotypes; however, the strawberry genotypes have not produced any seeds to understand inheritance of resistance in their F₁ and F₂ populations yet. The identified mildew resistances in the three wild strawberry ecotypes could be different genetics systems; however, it will be interesting and potentially useful for further studies. The wild diploid strawberry-GOU1 powdery mildew interactions suit a good host-pathogen interaction model with several advantages such as small genome size of wild strawberries, easy plant handling and massive seed production. It could be worth studying to identify resistance alleles that might be useful in breeding programs of commercial strawberries or they could be utilized to control for powdery mildew in other plant species (Hancock, and Luby, 1993).

The GOU1 powdery mildew isolate thrived exclusively on the ecotype 1, with sparse growth pattern on the ecotype 2 (Figure 2). However, the GOU1 powdery mildew cannot grow on ecotype 3. The pronounced susceptibilities and resistances among wild strawberries might reflect the relatively recent evolution of the wild diploid species with transposable elements. The transposable elements are major components of wild strawberry genomes (Shulaev *et al.*, 2010).

The results could be indicated that there is an individual monogenic resistance allele controls resistance mechanism to

the *Podosphaera aphanis* var. *aphanis* GOU1 isolate. The hypersensitive response generally occurred only in monogenic resistance, secondly in high humidity and low light conditions did not change the resistance response to GOU1. If the resistance mechanism is controlled by monogenic manner, the resistance will be confirmed when susceptible ecotype 1 and resistant ecotype 3 crossed together, the whole F₁ plants should be resistant to the GOU1 powdery mildew. This is strongly suggested in genetic studies (Shulaev *et al.*, 2010).

In vitro staining methods revealed that no mycelial growth and conidiophore production on resistant ecotype 3. The conidiospore penetration was associated with accumulation of hydrogen peroxide production on the host cells. Similar results were reported for different powdery mildew diseases (Adam & Somerville, 1996; Adam *et al.*, 1999; Xiao *et al.*, 1997). Our results suggest that wild diploid strawberry ecotype 3 is good resistance source to the powdery mildew GOU1 isolate.

Conclusions: Wild diploid strawberries are suitable plants to study host-pathogen interactions. Here, the identified powdery mildew is *Podosphaera aphanis* var. *aphanis* causes serious diseases not only on strawberries but also on other berries. To control powdery mildew is a challenge to manage where the resistant wild diploid strawberries provide good control to the pathogen. Further research is needed to determine the mechanism of wild strawberries' resistance to the powdery mildew.

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