THESIS

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GENETIC ANALYSIS OF PVY RESISTANCE IN POTATO

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1. INTRODUCTION

Potato is the 4th most-produced crop worldwide, a staple food for billions of people (WOOLFE and POATS 1987). Hence it is quixotic for scientists to address food security worldwide without studying this important crop and its history, potential future development, threats, and possible solutions. The introduction of potatoes significantly affected population growth in Europe, contributing to urbanization and development during the age of the industrial revolution. (NUNN and QIAN 2011).

The history of potato cultivation dates back to the pre-Columbian era (HAVERKORT et al. 2022), and at present it remains a vital part of global agriculture. From its origins in the Andean highlands of South America (GHYSELINCK et al. 2013), potato production has now spread to every continent. Its versatility, and nutritional value make it an important food source for billions of people. However, potato production faces significant challenges like pests, diseases, and climate change. To address these issues and ensure sustainable potato production (WANG et al. 2021), it is crucial to have a comprehensive understanding of the history, genetics, and significance of this crop.

Potato production incurs significant losses due to pests and pathogens. Potato production loss alone accounts for approximately 40% of total crop yield annually (OERKE 2006). Insects are one of the most important pests affecting potato crops, with the Colorado potato beetle being the most destructive defoliator (ALYOKHIN, et al. 2022). Nematodes, such as *Meloidogyne incognita* and *Ditylenchus destructor*, also cause huge yield losses (Fourie et al. 2017). Bacterial diseases, such as potato tuber soft rot and brown rot, affect potato production worldwide (CHARKOWSKI et al. 2020).

Potatoes are affected by various viruses transmitted through vectors or contact, leading to yield losses. More than 50 potato viruses have been reported, but PVY and PLRV are the most important (KREUZE et al. 2020). Traditional methods of controlling these viruses, such as pesticides and antibiotics, have raised concerns regarding effectiveness and environmental impact (CROWDER et al. 2010; MANYI-LOH et al. 2018). Integrated pest management (IPM) is a sustainable approach that combines biological, cultural, physical, and chemical tools to minimize economic, health, and environmental risks (JACOBSEN, et al. 2004).

Plant viruses significantly threaten global agricultural production and food security, particularly with the world's growing population. One such virus is PVY or potato virus Y, which was first reported by Smith in 1930 (SMITH 1931). PVY is a single-stranded positive RNA virus with a genome of approximately 10 kb (DOUGHERTY and CARRINGTON 1988). The Most dangerous strain of PVY is PVY^{NTN}, this strain was first reported in Hungary by Beczner and his colleagues (BECZNER, et al. 1984).

Resistance to plant viruses is a crucial factor in ensuring global food security. In the case of potato virus Y (PVY), various Solanum tuberosum varieties have shown resistance, with ten genes identified to date. Some of these genes are present in wild collections worldwide, while new ones continue to be discovered (SOLOMON-BLACKBURN and BARKER 2001). The genes' names are abbreviated as *Ry*, indicating resistance to PVY, and they include Ry_{sto} from *Solanum stoloniferum* and Ry_{adg} from *S. tuberosum* subs. *andigena*, and Ry_{chc} from *S. chacoense*. These genes have been localized on different chromosomes and are used by breeders to develop PVY-resistant potato varieties (CERNÁK et al. 2008; LI et al. 2022; SAGREDO et al. 2009).

The present study is part of a larger research project, where the general purpose is to explore the genetic background of resistance against PVY and *Phytophthora infestans,* the two major pathogens of

potatoes. In the gene bank of the Potato Research Centre at Keszthely a, collection of resistant genotypes to these two pathogens is available. In those genotypes the resistance genes derive from different, tuber-bearing wild potato species. Our final goal is to localize the analyzed resistance genes in the potato genome and to develop molecular tools, which then can be effectively used in resistance breeding of potatoes. For this end we use high-throughput molecular technologies with the following approaches: 1. Highly saturated maps of the potato genome are constructed based on 31,190 SNP markers which are detected by microarray analysis. 2. The haplotype-resolved whole genome sequence of the tetraploid cultivar White Lady is reconstructed from short (150 bp) Illumina reads and long (8,000 bp) PacBio HiFi reads. 3. For the genotypes with different resistance genes transcriptome datasets of samples taken before and after the infections are generated.

In the present study, we are focusing on the Ry_{adg} gene that conveys extreme resistance to PVY. The 89.415 potato genotype used in this study harbors the Ry_{adg} gene. A PVY sensitive variety, the *Somogyi kifli* is also used, and a 89.451 x *Somogyi kifli* segregating F1 population of 65 genotypes were created and analyzed for PVY resistance. Besides the parents also the 65 F1 genotypes were analyzed with microarray. Transcriptomes of the parental lines have been prepared from the samples immediately before infection and from the samples 24, 48 and 72 hours after infection. The transcripts of 89.451 were filtered with the *Somogyi kifli* transcripts and the most upregulated 100 transcripts of 89.451, which are not present in *Somogyi kifli* have been further analyzed. Also, similarity search with the available 607 complete coding sequence in the NCBI for resistance genes of the Solanum genus was performed on the 89.451 transcriptomes. In this study, results of the transcriptome analyses are presented.

2. LITERATURE OVERVIEW

2.1. Solanum tuberosum, historical background

Cultivated potato (*Solanum tuberosum*) is a heterozygous, tetraploid, globally important crop plant, producing high yields with high nutritional value (KUETE 2014; SCOTT and SUAREZ 2012). Potato originated approximately 10000 years ago in the Andean highlands in South America and was first cultivated in South America between three and seven thousand years ago. Since then, it has been a widespread crop all over the world (History Magazine - The Impact of the Potato n.d.).

This huge habitat range and distribution is most apparent in a paragraph from Darwin's log after his long beagle journey. He says, "It is remarkable that the same plant should be found on the sterile mountains of Central Chile, where a drop of rain does not fall for more than six months, and within the damp forests of the southern islands".

Mainly *S. tuberosum* is produced worldwide, although some related wild species are also grown in their native habitats. Potatoes are grown for their tubers which are enlarged portions of stolons developing under the ground. Nowadays, potato is the fourth most important food crop in the world after rice, wheat, and maize (HAVERKORT and STRUIK 2015).

2.2. Potato production

Potato is a staple food worldwide, with annual production estimated at 388,191,000 metric tons in 2017 (FAO 2019). During the last century, potato production increased by 20% (FAO 2019), and its production centers have changed.

In 1986, worldwide potato production was 309,000,000 tons per year. Most of it was produced in the industrialized countries, Europe, and the former USSR countries. Asia only accounted for 47,000,000

tons, or 12.1 percent of the world's production (1986 FAO production yearbook, 1987; The State of Food and Agriculture, 1990 n.d.). Asia now accounts for a larger share of global potato production than the industrialized countries. In the last half-century, potato production in Asia expanded by 120,000,000 tons (SCOTT and SUAREZ 2012), with China and India being the world's two biggest producers. Hence, the centralization of potato production in the world has shifted from Europe and America to Asia, or, in other words, from the developed countries to the developing countries. Besides this pattern change, consumption also increased more than twice as much in developing countries between 1960 and 2005 (JENNINGS et al. 2020).

Understanding the potential of potatoes for food safety worldwide requires secure production and the elimination of potential threats.

2.3. POTATO ECOPHYSIOLOGY

The high value of potatoes is not only due to their high diversity and productivity with relatively low costs but also because they are rich in nutrients such as carbohydrates (starch), minerals like potassium and magnesium, and vitamins (ZAHEER and AKHTAR 2016).

The ecophysiology of a certain crop is its physiological behavior in particular habitats (LÜTTGE and SCARANO 2004). In other words, it is the performance of the crop in terms of photosynthesis, respiration, growth, and nutrient uptake under certain environmental conditions such as the content of nutrients in the soil, the macro- and microclimate, stresses, temperature, light intensity, etc. (BEUKEMA and ZAAG 1990; RUNDEL, et al. 1994).

The potato is a C3 plant. This abbreviation indicates that this type of plant has a 3-carbon product as the first product of photosynthesis. The optimum temperature for the crop is between 20 and 25 °C;

at this temperature, the rate of photosynthesis is higher. The estimated required light for potato photosynthesis is around 7,8 hours per day, and for optimal tuber growth, shorter days are preferred.

Moreover, the physiological mechanism leading to the high starch content of the tubers is the process of carbohydrate assimilation via photosynthesis by the leaves; the vascular system then transports these carbohydrates to the tubers.

2.4. Pests and pathogens of potato

Potatoes are a food source for humans and other organisms. Each year, the world agricultural field incurs big losses due to the pests of crops. In potato production, losses are about 40 % of the total crop yield yearly (OERKE 2006).

2.4.1. Insects that infect potato

The most important insect pests that feed on potatoes are the aphids (*Myzus persicae),* which are vectors of *PVY* and other viruses, and the Colorado potato beetle (*Leptinotarsa decemlineata*), which can completely defoliate potato leaves and destroy the plants (ALYOKHIN n.d.). Other insect pests are wireworms (ALYOKHIN, et al. 2022).

2.4.2. Nematode pests of potato

Potatoes' most important nematode pests are root-knot parasitic nematodes such as *Meloidogyne incognita*, one of the four most common species in the world, or *Meloidogyne javanica*; the potato tuber rot nematode, *Ditylenchus destructor*; the potato cyst nematode *Globodera;* the spiral nematode *Helicotylenchus; etc.* (ABD-ELGAWAD 2020; FOURIE et al. 2017).

2.4.3. Bacterial diseases of potato

Bacterial pathogens also curb potato production. The six most important bacteria that affect potato crop production worldwide (CHARKOWSKI et al., 2020) are listed below.

The tuber soft rot disease is caused by *Pectobacterium,* previously known as *Erwinia carotowora,* which appears to be specific to potatoes (STEAD 1999). The brown rot is caused by *Ralstonia solanacearum, Dickeya* genus, which causes soft rot*; Clavibacter michiganensis,* which causes potato ring rot; *Streptomyces* species, which causes common scab; and *Candidatus liberibacter solanacearum,* which causes zebra chip in potatoes.

2.4.4. Fungal diseases of potato

Potatoes' most dangerous fungal pathogen *is Phytophthora infestans*, causing late blight disease, which induces up to 100 % tuber loss in the case of early infection (GOODWIN 1994). Yet there are a lot of other fungal threats for potatoes, like *Synchytrium endobioticum,* that cause wart disease. This pathogen was first reported in Hungary in 1985 (ABBAS, et al. 2013).

Other fungal infections include *Rhizoctonia solani, the* origin of black scurf disease, *Alternaria solani* which is responsible for early blight, and *Spongospora subterranea* the fungi behind root galling and tuber powdery scab diseases that result in quality and yield losses. *Spongospora subterranea* is also a vector of the potato mop-top virus.

2.5. Potato viruses

The viruses that affect potatoes are members of multiple viral families. *Potiviridae* (PVY), *Alphaflexiviridae* (PVX), *Comoviridae* (PBRV)*, Luteoviridae (*PLRV), etc. Viruses are transmitted to the plant either by vectors, by grafting, or by planting a seed that is not virus free. More than 50 potato viruses have been reported until now. However, some cause minor yield losses, while others can cause

up to 80 percent, like PVY and PLRV, the two most significant potato viruses (KREUZE et al. 2020). Further, some viruses depend on the potato to continue their cycle and spread, while others don't. This is mainly because of the difference in the number of host species of the virus (SALAZAR, 1996.).

For some viruses, the symptoms are characteristic, like the dark rings that are characteristic of the PVY virus. Others, like the mosaic symptoms, are more common for several virus types. Hence, their identification requires further analysis.

Threats to potato production and food security come from different directions and sources. It is necessary to have an integrated strategy to handle all these threats because, in most cases, pathogens and pests endanger production simultaneously. (RUBIO, et al. 2020).

Thus, we need to understand several possible diseases in *Solanum tuberosum* to guarantee the proper diagnosis, control, and treatment.

2.6. The control methods

Conventionally, the use of pesticides against fungi and insect-borne diseases or antibiotics against bacterial diseases is the dominant agricultural practice worldwide. Still, the development of resistance against pesticides and antibiotics raised concern for this method's effectiveness and environmental impact. For insects alone, it is estimated that the overuse of chemicals has led to the evolution of about 500 arthropod species resistant to one or more classes of insecticides. On the one hand, agrochemicals endanger biodiversity; on the other hand, they increase the costs of potato production. Because of these problems, there are more strict regulations on the use of pesticides in many countries (MANTZOUKAS and ELIOPOULOS 2020). All of these have led to the development of new plant pest control approaches. The most renowned is integrated pest management (IPM), a sustainable

approach to managing pests by combining biological, cultural, physical, and chemical tools to minimize economic, health, and environmental risks (JACOBSEN, et al. 2004).

IPM is actually in use, for PVY, the control by combining the mineral oil and crop border method limited PVY occurrence for 3 years out of 3 (BOITEAU, et al. 2009). IPM is effective and sustainable at the same time, it is a method that assists humans to tackle agricultural pests, bacteria, some bacterial species in the Pseudomonas and bacillus genera (SHODA 2000), are control agents against other pests or other bacterial species (JACOBSEN, et al. 2004).

Furthermore, biological control is a wide space for organisms interacting with each other in a way one organism can manipulate or stop the growth of another. In general, pathogens that parasitize other pathogens or pests are used for control in biological control. For example, endophytic fungi can control different insects or bacteriophages to control bacterial diseases.

Many studies have shown the importance of genetic control. WHITFIELD and ROTENBERG (2015) studied how genetic control can target not only the virus or bacteria but also the vector by the manipulation of functional proteins, it was possible to block their transmission. CRISPR-Cas9 has been studied and is also known for engineering resistance (CHANDRASEKARAN et al. 2016). Moreover, another study (KHAN et al. 2018) showed the perspective of CRISPR-Cas13a in targeting the RNA virus genome or the mRNA of the host. RNA silencing and post-transcriptional gene silencing can also be utilized to develop resistant plants (NEUPANE et al. 2019).

Finally, the resistance genes of wild potato species are highly important for the studies of resistance response to biotic stresses (DE RONDE, et al. 2014). This approach of genetic control is a promising, sustainable, and low-cost way to tackle pests and pathogens by breeding resistant varieties.

2.7.PVY

Plant viruses threaten agricultural production and food security, especially with the world's increasing population. PVY, or Potato Virus Y, was reported for the first time in 1930 by Smith (SMITH, 1931). It is a single-stranded positive RNA virus with a genome of approximately 10 kb. This genome is translated into a single polyprotein, later cleaved into ten multifunctional proteins (DOUGHERTY and CARRINGTON 1988).

PVY is one of the most important viruses affecting potato crop production; 30–64% yield losses have been reported after the crop is infected or grown from infected seeds. (VALKONEN and VALKONEN 2007), PVY is ranked in the top 10 plant viruses in molecular plant pathology (GLASA et al., 2021; LACOMME and JACQUOT, 2017; SCHOLTHOF et al., 2011).

PVY belongs to the family *Potyviradea* and has a wide host plant range; 495 species in 72 genera from 31 families were reported to be infected by PVY (EL-AZIZ 2020). After the first identification of PVY, several strains have been subscribed, the five major strain groups are PVYO, PVYN, PVYC, PVYZ, and PVYE (KERLAN et al. 2011), additionally, other recombinant isolates have been recognized these isolates are composed of segments of different strains, these isolates include PVY^{NTN} and $PVY^{N\text{-}wi}$ (TORRANCE and TALIANKSY 2020), PVY^{NTN} genotype has been reported in many areas of the world and are a major concern for potato production (DELLA BARTOLA, et al. 2020) because it induces severe symptoms in potato tubers known as potato tuber necrotic ringspot disease, furthermore, sub strain of PVY0 was reported which is distinguished from the original strain serologically, that is PVY0-05 (KARASEV et al. 2011).

Overall, PVY has several strains and recombinants that constitute a challenge to modern-day agriculture, especially the challenge of detecting in the early stages of infection what exact strain the farmer is dealing with.

2.7.1 PVY detection

The diagnosis of PVY and its various strains varies from simple symptom-based to molecular diagnoses. A symptom-based diagnosis is usually the first step of diagnosis, but it may not be accurate knowing that many pathogens can cause the same symptoms, like mosaic leaves, leaf drops, necrotic patterns, etc. (FUNKE et al. 2017).

The molecular diagnosis is more definite; Elisa is a method of detection where a viral protein has been used traditionally to diagnose and quantify PVY incidence in a crop or seed lot (MACKENZIE, et al. 2015). Another molecular method for PVY detection is PCR, with variants such as reverse transcriptome PCR RT-PCR or multiplex PCR. Multiplex PCR is used due to its efficiency in rapidly identifying the genotype and quantifying multiple DNA targets simultaneously in a single reaction, in addition to its reduced cost compared to RT-PCR. In contrast, the immunocapture reverse transcriptase PCR was more sensitive in detecting PVY in infected plant sap than multiplex RT-PCR or ELISA alone while retaining the ability to differentiate strains that can infect potatoes. Furthermore, novel methods in PVY identification have been indicated by using Taqman chemistry with higher sensitivity than conventional reverse transcription (KHELIFA 2019; MALLIK, et al. 2012; Multiplex Real-Time PCR n.d.).

Moreover, methods that incorporate both the Elisa test and PCR have been studied and successfully detected normal and mixed strains accurately and sensitively. The detection could be done in the vector of the virus, *Myzus persicae*, or plant hosts.

2.7.2 Resistance Breeding for PVY

Breeding for resistance in arable plants is an activity that has its roots in the ancient history of agriculture. Although it was based on phenotypical appearance rather than genetic identification, it was a way for farmers to distinguish between their cultivars that are more resistant or susceptible to pathogens. The Greek philosopher Theophrastus noted in the third century B.C. that cultivated varieties differed in their ability to avoid disease (INFO 2017).

Resistance breeding in agriculture has been studied scientifically much more extensively in the last century. Notable scientific studies in this field date back more than 100 years (ORTON 1918), and potatoes, as important staple food, have had their fair share of these studies. In Europe, the Scottish William Patterson probably did the first systematic effort to breed potatoes for variety development, and his effort resulted in the cultivar Victoria in 1856 (STUART and COMPANY 2019). Moreover, in 1910, the US Department of Agriculture initiated a potato breeding program focusing on breeding for virus resistance (JANSKY and SPOONER 2018). Breeding approaches for potatoes are diverse, discussed in (JANSKY and SPOONER 2018). However, the main approach is based on cross-parental breeding. The advantages of resistance breeding for the virus are huge, both economically and environmentally, since resistance varieties give higher yields to ensure food security and give us an escape from the overuse of chemical pesticides that can have an environmental effect.

Nowadays, we know two types of resistance in *Solanum tuberosum:* extreme resistance (ER) and hypersensitive resistance (HR). They are described by (SOLOMON-BLACKBURN and BARKER 2001). In the case of ER, potato plants show no symptoms or minimal necrosis in some genotypes (BAEBLER, et al. 2020), while in the case of HR, visible areas of necrosis because of programmed cell death develop in inoculated leaves with PVY (TORRANCE and TALIANKSY 2020).

2.8. PVY Resistance genes in Solanum

Ten genes in different *Solanum tuberosum* varieties are associated with resistance to PVY. Some resistance genes are available in various collections of wild species, whilst new ones are being discovered, and there are probably many others yet to be discovered (Solomon-Blackburn and Barker 2001).

In the potato crop, the widest group of dominant resistant genes to viruses falls into the class of nucleotide binding site-leucine rich repeat (NBS-LRR), a sequence motif typical for resistance response. (MAULE, et al. 2007).

Additionally, there are special types of genes that are candidates to be resistant: plant resistance gene analogs, or RGAs, with conserved domains and motifs that play specific roles in resistance (SEKHWAL et al. 2015).

The abbreviated names for the genes are RY, indicating the resistance in R, and PVY, abbreviated with Y. One of the most studied genes conferring extreme resistance is RYsto, which originated from the *Solanum stoloniferum* variety. Rysto was marked to chromosome XII in potatoes (ISTVÁN CERNÁK et al. 2008; GRECH-BARAN et al. 2020; KONDRÁK et al. 2020; LACOMME and JACQUOT 2017).

What's more, the closest marker to the gene obtained by marker-assisted selection MAS was located at 0.53 cM from the gene, but it did not have any diagnostic value; the closest one to the gene with a diagnostic value was located at 2.95 cM from the gene (I. CERNÁK et al. 2008). Another gene conferring extreme resistance to PVY is Ryadg, originating from *Solanum tuberosum* group Andigena; it has been localized on chromosome XI (SAGREDO et al. 2009; SONG 2004). Rychc also confers extreme resistance to all known strains, and it is used with previously mentioned genes by breeders to develop varieties resistant to PVY (HERRERA et al. 2018). It originates from *Solanum chacoense*, which

is a wild species of potato that is genetically diploid (LI et al. 2022), and it was mapped on chromosome 9 to a 2.4MB region (GREGORY L. ELISON et al. 2020; SATO et al. 2006).

2.9. Next generation sequencing

The term next-generation sequencing (NGS) is more than 16 years old, but it is still used colloquially together with massively parallel or deep sequencing terms to describe a DNA sequencing technology that has revolutionized genomic research (BEHJATI and TARPEY 2013). All NGS platforms perform the parallel sequencing of millions of small fragments of DNA. Bioinformatics analyses are used to piece together these fragments by mapping the individual reads to the reference genome.

Based on the read length of DNA, there are two main approaches: the short-read approach and the long-read approach. Illumina technology presents the first approach (LEVY and MYERS 2016). For example, Illumina HiSeq and MiSeq platforms are commonly used to study microbial communities. Still, they exhibit differences in terms of the length and quantity of reads generated, according to (CAPORASO et al. 2012). There is a significant difference in scale between the HiSeq and MiSeq platforms. The HiSeq2000 can produce over 50 Gb of data daily, generating 1.6 billion 100-base paired-end reads in a 10.8-day run. On the other hand, the MiSeq is designed for single-day experiments and produces 1.5 Gb of data per day from 5 million 150-base paired-end reads**.**

Currently, NGS is used in agricultural research to study the genetic potential of plants for resistance to diseases or resilience to stresses. This technology provides massive genomic data, and it is efficient, rapid, and can be performed at a relatively low cost compared to the traditional Sanger sequencing method (SANGER, et al. 1977). NGS can unveil resistance potentials in wild species and domesticated ones (HU et al. 2021). From wild relatives, we can obtain species-specific data by whole-genome shotgun sequencing.

This sequence data can be used to design species-specific polymerase chain reaction (PCR) primers (ASHRAF et al. 2022; CAPORASO et al. 2012).

Finally, NGS is a huge step in pushing crop improvement forward because it is applicable, accurate, and low-cost.

3. MATERIALS AND METHODS

3.1. Plant materials

In middle of April 2022, tubers of the 56 F1 genotypes of the 89.451 x *Somogyi kifli* cross were planted in peat in 5 L pots and grown under a plastic roof. From each genotype three tubers were planted in separate pots, i.e. the experiment was done in three replications. Watering was applied daily, and chemical protection against insect vectors was done when necessary.

The parents 89.451 and *Somogyi kifli* were grown in five replications in a phytotron under conditions revealed in Table 1.

Table.1 Program of the phytotron for growing the parental lines.

Hours of the day when values are changed	Light intensity[1] (%)	Temperature (˚C)	Humidity (%)	Air flow[2] (%)
4	0	20	65	30
5	40	21	60	30
6	60	22	58	30
7	80	23	56	30
8	85	24	54	30
9	90	25	52	30
10	95	25	50	30
16	85	25	52	30
17	80	24	54	30
18	75	23	56	30

19	70	22	58	30
20	50	21	60	30
21	30	20	60	30
22	0	20	60	30

Notes: 1: 3,000 lux is 100%; 2: percentage of the maximum air flow.

3.2. PVY infection tests

The PVY-NTN-H substrain was used for the infection, the isolate was maintained on a susceptible host, *Nicotiana tabacum* Xanthi-nc. After the observation of infection symptoms, the leaves of the infected host (tobacco) were harvested, chopped, and mashed in a mortar with phosphate buffer saline solution (PBS). The resulting liquid solution was filtered through two gauze sheets.

The virus solution was sprinkled with silicon carbide (SiC), carborundum powder on 50% of the adaxial surface of the largest leaves on the young potato cultivars. The rubbing of the virus solution onto the leaves was done with a glass spatula. Both the parental lines and the segregating population were infected, and six weeks later the symptoms were observed. A sampling of uninfected leaflets from infected leaves was done.

3.3. Virus detection

For the virus detection in the leaflets samples, the DAS-ElISA method was performed, the AP-PAbs (alkalic-phosphatase conjugated PAbs9 antibody) (Loewe Biochemica GmbH, Germany) was used with Para-Nitrophenyl-phosphate and polystyrene plates, and the producer protocol was followed. (Corning 96-Well EIA/RIA Clear Flat Bottom Polystyrene High Band Microplate, USA).

For the positive control, *Nicotiana tabacum* Xanthi-nc leaf juice was used, while for the negative control, sterile *in vitro* potato plants were utilized.

The extinction values were measured for the samples at 405 nm on a BMG Labtech Spectrostar Nano photometer (BMG Labtech, Germany).

3.4. RNA extraction

Samples from uninfected leaflets of infected leaves were taken for transcriptome analyses at six different time points (2, 4, 6, 24, 48, and 72 hours) after infection, and one sample was taken for control immediately before infection.

The leaf samples were collected on dry ice and stored at -80°C until RNA extraction. The RNA extraction was done by the TRIzol method (CHOMCZYNSKI and SACCHI 1987), using the Direct-zol RNA Microprep Kit (Zymo Research, USA), following the protocol of the producer. Fifty mg of leaf samples were smashed in 2 mL tubes in the presence of 100 μL TRIzol using a DNA mill (Retsch, Germany). Then, 400 μL of TRIzol was added, and total RNA was extracted.

Afterward, Poly-A enrichment was done with the Poly(A) RNA Selection Kit (Lexogen, Austria). To check the RNA integrity value, the 2100 Bioanalyzer (Agilent, USA) and the RNA 6000 Pico kit were utilized. For further processing, RNA with a RIN value higher than 5.5 was used.

3.5. Library preparations and transcriptome sequencings

The transcriptome sequencing libraries were prepared with the NEXTFLEX Rapid Directional RNA-Seq Kit 2.0 (PerkinElmer, USA), according to the producer's guidelines.

Transcriptome sequencing using a NextSeq 500 (Illumina, USA) type sequencer was done for the control and the 24th, 48th, and 72nd hours samples after treatment of White Lady, *Somogyi kifli* (S), and the breeding line 89.451.

3.6. Identification of differentially expressed genes (DEGs)

Raw Illumina reads obtained from the RNAseq were processed for standard quality controls and cleaned with Trimmomatic (Bolger, et al. 2014) to remove contaminant residual adapters. The counts of mapped reads per gene per sample were obtained with Salmon (Patro et al. 2017). Then, the DESeq2 package (LOVE, et al. 2014) was applied to identify differentially expressed genes (DEGs) in the infected samples using R version 4.2.2 (R: The R Project for Statistical Computing, n.d.). To control the false discovery rate (FDR), the resulting p-values were adjusted according to Benjamini and Hochberg's approach (Benjamini and Hochberg 1995), where an adjusted $p < 0.05$ is accepted. The genes with an absolute log2 fold change ≥1 were considered to be DEGs.

3.7. Identification of PVY infection-related genes

Differential expression was visualized using the EnhancedVolcano and ggplot2 packages, and the transcripts showing the largest overexpression were filtered in R version 4.2.2 (R: The R Project for Statistical Computing, n.d.). Sequences for the transcripts were obtained from the reference transcriptome using custom scripts and the SeqKit program (SHEN et al. 2016), and then a similarity search was done in the nucleotide database of NCBI using BLASTN.

Reads were aligned to a White Lady haplotype resolved transcriptome reference sequence. For that White Lady transcriptome constructed from short Illumina and long PacBio HiFi reads was used. The merit of this approach is, that a long read can cover the entire length of a transcript, and reliability of

the sequence is assured by the percise of the short read generating SbS technology. Hence, the different allelic and alternative versions of the same gene can be detected in the same reaction.

The most upregulated 100 transcripts of 89.451 which are not present in the PVY sensible *Somogyi kifli* were selected and annotated in the NCBI.

The complete coding sequence of 609 resistance genes identified in the Solanum genus were downloaded from the NCBI and the transcriptome datasets were screened for sequence similarity.

4. RESULTS AND THEIR ASSESSMENT

4.1. Genes upregulation

In total, 16,000 transcripts were identified in PVY-resistant breeding line 89.451 but are missing from the sensitive cultivar *Somogyi kifli*. From these genes 9,000 were upregulated, their expression increased significant after infection. The magnitude of the upregulation is illustrated in Figures 1 and 2.

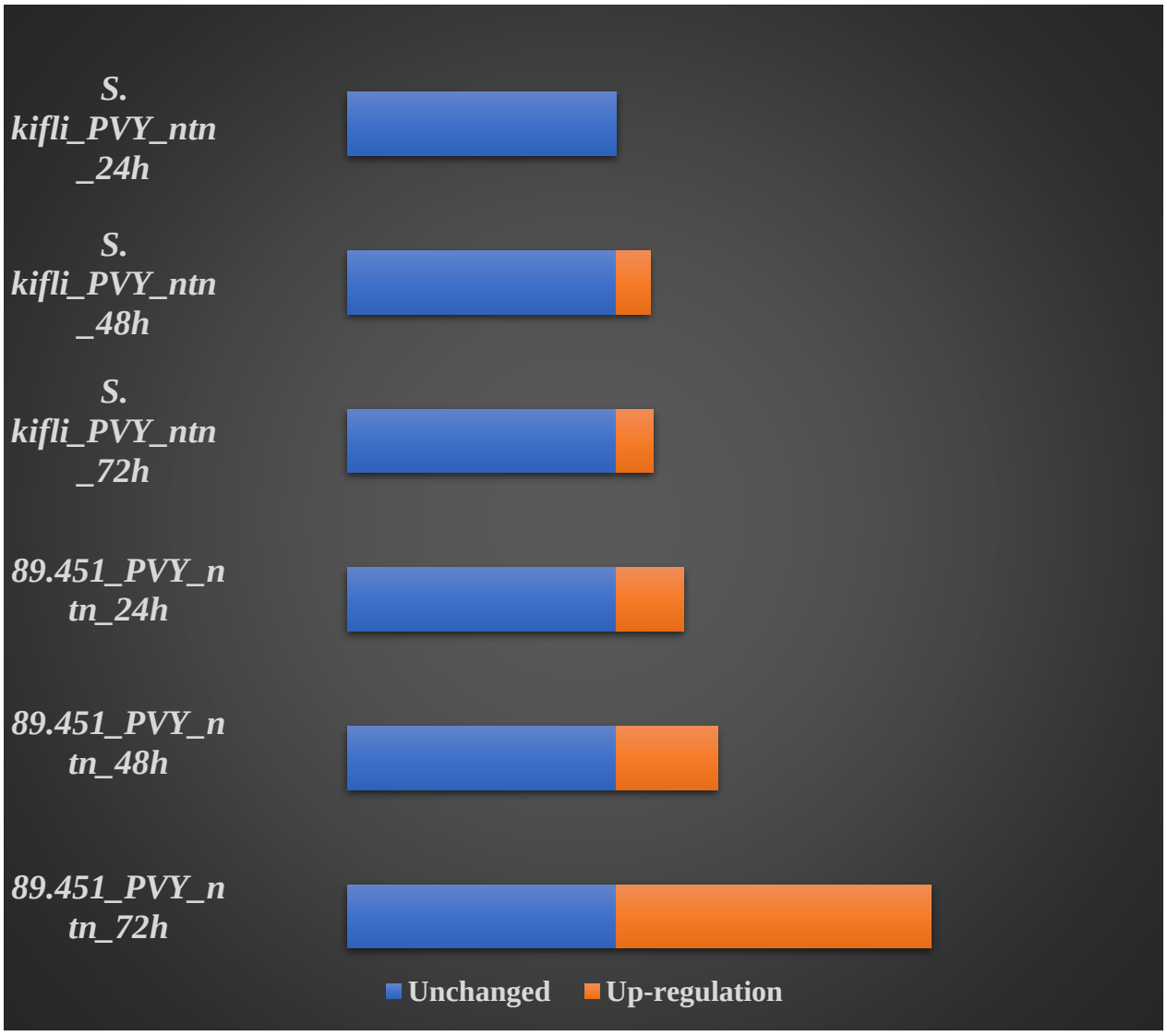

Figure 1. Identification of differentially expressed genes (DEGs), with an absolute log2 fold change ≥1.

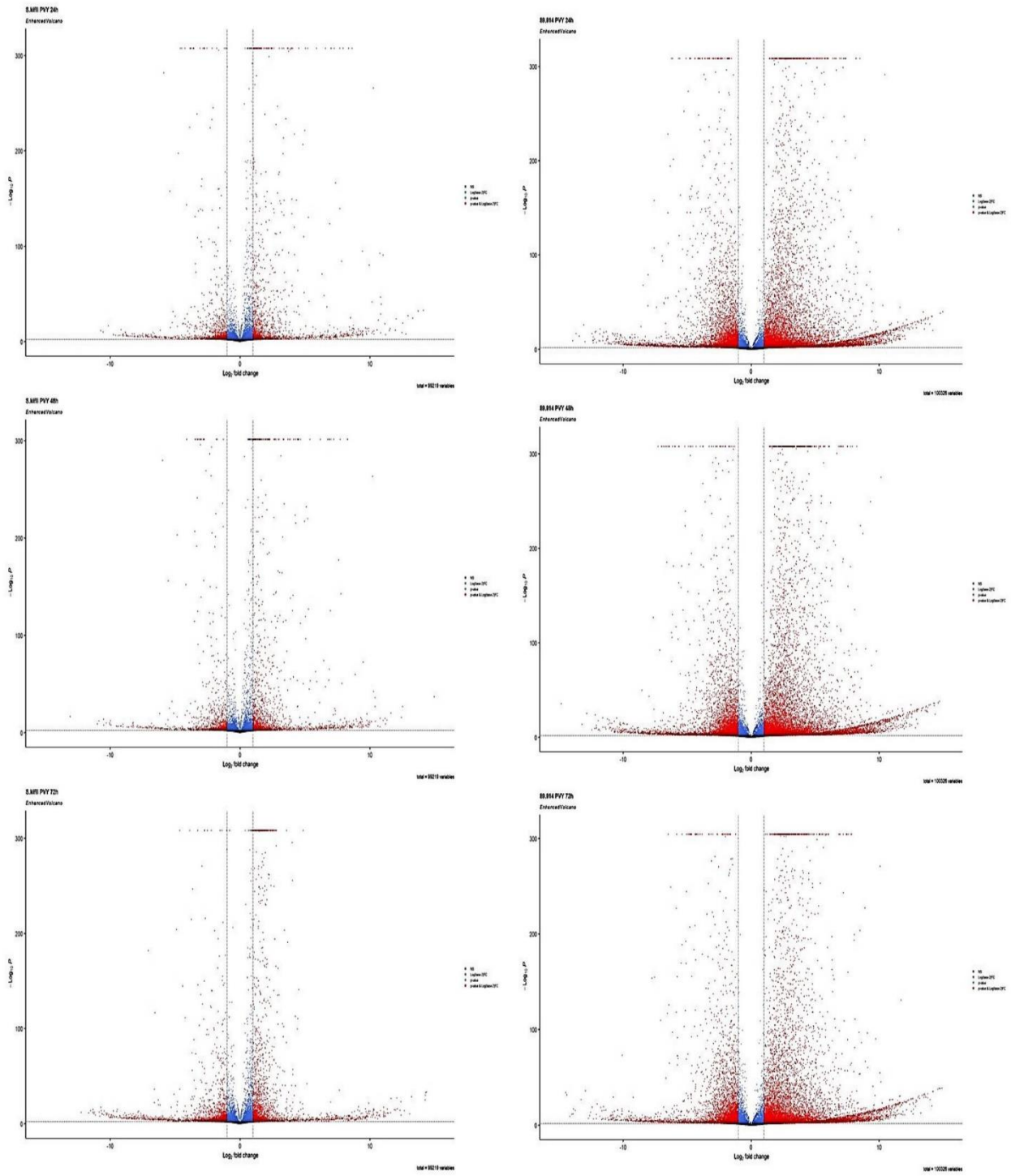

Figure 2. Identification of differentially expressed genes (DEGs), with an absolute log2 fold change ≥1 in EnhancedVolcano.

Among the most upregulated 100 transcripts of 89.451, only two transcripts (XM_006347250.2; XM_006361767.2) were identical, i.e., these possessed 100% similarity and 100 query coverage with genes in the NCBI. However, they are not involved in the resistance response, but they have metabolic functions. (Nam 2022; RFS1 - Probable galactinol--sucrose galactosyltransferase 1 - Arabidopsis thaliana (Mouse-ear cress) | UniProtKB | UniProt n.d.). Other transcript similarity tests for percentage identity varied from 85.07% to 99.92%, while for the query coverage, it varied from 5 to 99%.

4.2 Similarity search with resistance genes of the Solanum genus

Resistance gene sequences of the Solanum genus have been collected from the NCBI database. In total, the complete coding sequence (CDS) of 609 genes could be identified. These sequences were downloaded and a similarity search in the transcriptomes of 89.451 and Somogyi kifli was performed.

Out of the 609 resistance genes of the Solanum genus, 347 showed 82-100% similarity with transcripts of the 89.451 line, while 340 with transcripts of the Somogyi kifli. These relatively high numbers of transcripts and high percentage of sequence similarity in the two genotypes indicate the abundance of resistance gene like sequences in potato. Further, in 89.451 the similarity search resulted 27 unique hits present in 89.451 breeding line, but absent int the variety Somogyi kifli. These genes were checked in "UniProt" database for further characterization, and the results are presented in Table.2.

Table 2. The characterization of unique hits in 89.451.

UniProt ID	Protein name	Gene name	Organism	Len-gth	Gene Ontology	KEGG cross-reference	Percentage identity	Score	E value
M1B976	Tm-1^GCR26 protein	102594208	*Solanum tuberosum* (Potato)	754 AA	catalytic activity	sot:102594208	96.4	3699	0
A0A3Q7FPY9	Peptidase C1A*	N/A	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	654 AA	extracellular space, lysosome, cysteine-type endopeptidase activity, proteolysis involved in protein catabolic process	N/A	100	1818	0
Q71BH0^	ToMV resistance protein Tm-2(GCR236), Disease resistance protein Tm-2	Tm-2	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	861 AA	Inhibitor of viral movements which confers resistance to some tomato viruses including tomato mosaic virus (ToMV) (e.g. isolates L, W3 and SL-1) and tobacco mosaic virus*	N/A	100	4459	0
Q40235	Receptor-like protein Cf-9	CF-9	*Solanum pimpinellifolium* (Currant tomato) (*Lycopersicon pimpinellifolium*	863 AA	plasma membrane, defense response to fungus, response to molecule of fungal origin	ag:AAA65235	99	4452	0
P93215	Pto kinase, Serine/threonine protein kinase Pto	pto, 101268866	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	311 AA	plasma membrane, ATP binding, protein kinase activity, protein serine/threonine kinase activity, transmembrane receptor protein tyrosine kinase activity, protein	sly:101268866	83.8	1340	0

M0ZKX2	Allene oxide synthase 2	102577479	*Solanum tuberosum* (Potato)	510 AA	autophosphorylation heme binding, iron ion binding, monooxygenase activity, oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, sterol metabolic process	sot:102577479	97.6	2596	0
M1A6Z2	Disease resistance protein SlVe2	102605363	*Solanum tuberosum* (Potato)	1,138 AA	plasma membrane, defense response to fungus	sot:102605363	85.4	1765	0
K4BPC1	Putative PVX disease resistance*	N/A	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	836 AA	ADP binding, defense response to another organism	N/A	83.6	3576	0
C1KBP2	Invertase inhibitor, Vacuolar invertase inhibitor	INH2alpha, 102586828, inh2	*Solanum tuberosum* (Potato)	178 AA	extracellular region, enzyme inhibitor activity, hydrolase activity, negative regulation of catalytic activity	sot:102586828	97.8	892	3.50E-121
M1B4U8	AP2 domain CBF protein	N/A	*Solanum tuberosum* (Potato)	258 AA	nucleus, DNA binding, DNA-binding transcription factor activity, defense response	N/A	73.2	909	7.70E-122
M1ADC5	Disease resistance protein Gpa2	102579866	*Solanum tuberosum* (Potato)	901 AA	ADP binding, a defense response to another organism.	sot:102579866	88.9	4129	0
R4HYA4^	Eukaryotic translation initiation factor	eIF4E-eva1	*Solanum etuberosum* (Wild potato)	231 AA	Component of the protein complex eIF4F, which is	N/A	100	1254	1.10E-174

	4E allele Eva1, eIF4E-Eva1, eIF-4F 25 kDa subunit, eIF-4F p26 subunit, eIF4E-1 variant 1, mRNA cap-binding protein				involved in the recognition of the mRNA cap, ATP-dependent unwinding of 5'-terminal secondary structure and recruitment				
K4BWG3	Matrix metalloproteinases protein 4	101252585	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	357 AA	extracellular matrix, metalloid peptidase activity, zinc ion binding, collagen catabolic process, extracellular matrix organization, proteolysis	sly:101252585	100	1899	0.00E+00
P93216	Serine/threonine protein kinase Fen	fen, 101263706	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	320 AA	plasma membrane, ATP binding, protein kinase activity, protein serine/threonine kinase activity, transmembrane receptor protein tyrosine kinase activity, protein autophosphorylation	sly:101263706	97.5	1637	0.00E+00
A0A3Q7ERL2	Protein kinase domain-containing protein	N/A	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	715 AA	plasma membrane, ATP binding, carbohydrate binding, transmembrane receptor protein serine/threonine kinase activity, defense response to bacterium, defense response to	N/A	100	3705	0.00E+00

					oomycetes, protein phosphorylation				
O49879	Receptor-like protein Cf-9 homolog	HCR9-0, CF-0, Solyc01g006550.2.1	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	845 AA	plasma membrane	sly:101247650	90.4	4013	0.00E+00
A0A1D8EJF9 ^	Eukaryotic translation initiation factor 4E-1, eIF4E-1, eIF-4F 25 kDa subunit, eIF-4F p26 subunit, mRNA cap-binding protein	Eif4e1	*Solanum pimpinellifolium* (Currant tomato) (*Lycopersicon pimpinellifoliu*)	231 AA	Component of the protein complex eIF4F, which is involved in the recognition of the mRNA cap, ATP-dependent unwinding of 5'-terminal secondary structure and recruitment*	N/A	99.6	1249	6.50E-174
A0A3Q7IA72	NB-ARC domain-containing protein	N/A	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	1,354 AA	ADP binding, defense response to another organism	N/A	94.3	5970	0.00E+00
A0A3Q7IA72	NB-ARC domain-containing protein	N/A	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	1,354 AA	ADP binding, defense response to another organism	N/A	93.6	5939	0.00E+00
A0A3Q7IA73	NB-ARC domain-containing protein	N/A	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	1,354 AA	ADP binding, defense response to another organism	N/A	94.2	5977	0.00E+00
A0A3Q7JPR0	carbonic anhydrase, 4.2.1.1	N/A	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	1,266 AA	carbonate dehydratase activity, hydro-lyase activity, zinc ion binding, defense response to other organism,	N/A	96.6	4601	0.00E+00

					one-carbon metabolic process				
A0A3Q7GTH3	OS11G0199801 PROTEIN*	101243720	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	272 AA	N/A	sly:101243720	100	1420	0.00E+00
A0A3Q7GR60	NB-ARC domain-containing protein	N/A	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	888 AA	ADP binding, defense response to other organism	N/A	72.6	3221	0.00E+00
A0A3Q7GR60	NB-ARC domain-containing protein	N/A	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	889 AA	ADP binding, defense response to other organism	N/A	72.3	3200	0.00E+00
P93215	Pto kinase, Serine/threonine protein kinase Pto	pto, 101268866	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	311 AA	plasma membrane, ATP binding, protein kinase activity, protein serine/threonine kinase activity, transmembrane receptor protein tyrosine kinase activity, protein autophosphorylation	sly:101268866	84.1	1342	0.00E+00
A0A3Q7GH73	Protein kinase domain-containing protein	109120256	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	151 AA	ATP binding. transmembrane receptor protein tyrosine kinase activity	sly:109120256	89.6	530	4.60E-67
Q9LLC7	LescPth2	101264008	*Solanum lycopersicum* (Tomato) (*Lycopersicon esculentum*)	323 AA	plasma membrane, ATP binding, protein kinase activity	sly:101264008	93.7	847	3.30E-112

(*) refers to the outputs of the "InterPro" database, and (^) refers to the viral resistance-related genes. AA – amino acids; N/A – no data.

4.3. Plant virus resistant genes

Based on the search results of 89.451 unique hits, we could identify two genes that are related to plant virus resistance.: 1- The Tomato mosaic virus resistance gene Tm-2(2). It is located on the chromosome 9 in *Solanum lycopersicum*. The gene belongs to the class of plant resistance (R) genes known as coiled-coil/nucleotide binding-ARC/leucine-rich repeat (LRR) proteins, and imparts resistance to the tomato mosaic virus (ToMV) (Kobayashi et al. 2011). 2- The translation initiation factor 4E(elF4e), this gene has been implicated in naturally occurring resistance to Potato virus Y (PVY) in pepper and in its synthenic gene, Pto-1 in tomato (Ruffel et al. 2005). Remarkably both genes showed 100% identity in UniProt sequence.

The uncharacterized proteins were analyzed in InterPro to check their putative functions. Further advancement in the annotation is in progress. This is done by the elimination of sequences with low similarity or irrelevant functions, and the incorporation of candidate genes for additional analysis.

4.4. Common hits assessment

From all hits, the highest number of nucleotides aligned from the transcript and the 609 resistance genes was 4402 (U65391.1), which corresponded to the "*Lycopersicon esculentum* PRF gene". Analysis in UniProt shows that this gene confers resistance to late blight. Moreover, in the common hits an alignment number of 3937 corresponded to "*Solanum tuberosum* subclone SH27O14e CC-NBS-LRR protein gene" (EF638455.1). Since this gene belongs to the NBS-LRR class, we consider it to be a more relevant candidate for viral resistance. Also, in the commonly expressed genes between the two genotypes, similarity of 95.258% and 3606 nucleotides was identified with the PVY resistance gene *Ry**chc*** in *Solanum tuberosum* (LC726345.1).

4.5. Orthologue genes investigation

The process of transcriptome alignment with the 609 Solanum-resistant genes also relies on resistance orthologue gene searches. The likelihood of orthologue genes, which are genes that originate by speciation and have the same function (GABALDÓN and KOONIN 2013) is high in closely related species, and there can be thousands of orthologue genes, as was shown by HIRAKAWA et al. (2014) in eggplant and tomato genome comparison. For resistance, the study of late blight-resistant orthologue genes in wild species provides an example (LIU and HALTERMAN 2006).

Moreover, the approach in the ongoing study is also considerable with the possibility of the same gene conferring resistance to different pests or pathogens, even as mentioned before in different species, which could be due to typical resistance motifs with variations during divergence from a common ancestor. The work of ROSSI et al. (1998) demonstrated that the nematode resistance gene Mi, also conferred resistance against potato aphids, two organisms belonging to separate phyla. The prediction of gene sequences that are not 100% homologous in the NCBI database is a tangible application towards the annotation of the genes, this could be done using Augustus software (STANKE and MORGENSTERN 2005) to predict the structures and functions of the assumed novel genes.

5. CONCLUSION AND SUGGESTIONS

The present study delivered insights on the genetic response of two different potato cultivars following infection with PVY, the resistant cultivar 89.451 possessed 16000 transcripts that are absent in the sensitive cultivar *Somogyi kifli*. Out of these, 9000 transcripts were found to be upregulated. The similarity alignment of the most 100 upregulated transcripts was done to annotate the genes. Further comparison of the transcriptome with the 609 Solanum-resistant genes present in the NCBI database resulted in the selection of 26 unique hits to the resistant cultivar out of which two were found to have virus resistance function with 100% alignment.

This study is part of a carried on scientific project aimed on unveiling the genetic background of PVY resistance and tracking of resistant gene Ry_{adg}. This project and the procedure of annotating the gene is still ongoing, hereby final annotation of the genes is yet to be done. Some approaches based on screening motifs that are typical to virus resistance in potato such as NBS-LRR and gene annotation show a promising course. Based on this we can recommend further progression in the process of assigning the assumed functions for each transcript by gene annotation. The ultimate aim of isolating Ry_{adg} is a process that exceeds time limit of this Master's program.

Furthermore, we recommend the investigation of the 13 selected genes in an elimination procedure in order to reach the target gene that could be used in PVY resistance breeding programs. The 89.451 genome is suitable to investigate the genetic response. Few studies have been done in construction of haplotype resolved whole genome, the opportunity of having done this at Keszthely is providing a prospect to use it in comparison with other cultivars. Lastly, it is possible to study how the selected genes interact by molecular docking technique, to have their functions better understood.

6. SUMMARY

Potato is a globally important crop that provides food to billions of people. This crop is affected by various pests and diseases, and one of the most important diseases affecting potatoes is PVY. PVY is a single-stranded RNA virus transmitted mainly by aphids and can cause yield losses in potatoes of up to 80%. To control PVY, chemical spraying of the vectors is the conventional method. However, other approaches, such as Integrated Pest Management are becoming more popular. Resistance genes to PVY has been reported in many Solanum cultivars, such as *Solanum stoloniferum, Solanum tuberosum* subs. *andigena*, and *Solanum chacoense*. Most resistance genes belong to the family of NBS-LRR. In this study, we used Next Generation Sequencing to discover the genetic background of resistance to PVY in the 89.451 cultivar. Next Generation sequencing allows the sequencing of millions of small fragments of DNA.

Parental lines of potato were grown in 5 replications in a phytotron under controlled biotic and abiotic conditions in Keszthely. Tubers from the crossing between 89.451 and *Somogyi kifli* were planted in peat pots, and the experiment was replicated three times. The PVY-NTN-H isolate was used for the infection. The virus was maintained on a susceptible host (*Nicotiana tabacum-Xanthi-nc*) and the leaf juice of this host was used for positive control, while in vitro plants were used for negative control. The contamination was done mechanically by rubbing the virus solution with silicon carbide on the potatoes. After observation of the symptoms, virus detection was done by the DAS-ELISA test. RNA extraction was done by the TRIzol method using the Direct-zol RNA Microprep Kit. RNA with a RIN value higher than 5.5 was further used. Sequencing libraries were prepared, and sequencing was done using the NextSeq 500 (Illumina, USA) type sequencer present in the Bioinnovation centre at

Georgikon campus. The control and the 24th, 48th, and 72nd hour samples after treatment of White Lady, *Somogyi kifli (*S), and the breeding line 89.451 were sequenced. Afterward, differentially expressed genes were identified using the DESeq2 package and visualized through the EnhancedVolcano and ggplot2 packages. The White Lady haplotype resolved transcriptome, which is constructed from short Illumina and long PacBio HiFi reads, was used as a reference sequence to further identify infection-related genes. This approach is meritorious since the different allelic versions of the gene can be detected in the same reaction. Primary results were coherent with the research expectations; a total of 16000 transcripts were present in the PVY-resistant breeding line 89.451 while absent in the sensitive cultivar Somogyi kifli. 9000 of them were upregulated. The 100 most upregulated transcripts were selected and annotated using the NCBI Blast tool. Moreover, the transcriptome datasets were screened for similarity with the 609 Solanum genus resistance genes present in the NCBI. 26 unique hits to the resistant genotype, two of which were found to be associated with plants resistance to viral infections. Further studies for the annotation of the genes are ongoing, and the isolation of the Ry_{adg} gene is the goal of this project. 89.451 genome shows promising results in the path of the Ry_{adg} gene. We believe that additional studies are needed to assert the function of any gene such as gene knock-out test.

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As primary supervisor of the author of this thesis, I hereby declare that review of the thesis was done thoroughly; student was informed and guided on the method of citing literature sources in the dissertation, attention was drawn on the importance of using literature data in accordance with the relevant legal and ethical rules.

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STUDENT DECLARATION

Signed below, SIRAJEL MASRI, student of the Georgikon Campus of the Hungarian University of Agriculture and Life Science, at the BSc/MSc Course of Plant protection declare that the present Thesis is my own work and I have used the cited and quoted literature in accordance with the relevant legal and ethical rules. I understand that the one-page-summary of my thesis will be uploaded on the website of the Campus/Institute/Course and my Thesis will be available at the Host Department/Institute and in the repository of the University in accordance with the relevant legal and ethical rules.

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