BIOLICAL ROLE OF *GRAPEVINE FANLEAF VIRUS* (GFLV) IN WINEGROWING REGION OF NORTHERN PRIMORSKA

DISSERTATION

Anastazija JEŽ KREBELJ

Mentors:
Assist. Prof. Maruša Pompe Novak, Ph.D.
Assos. Prof. Paolo Sivilotti, Ph.D.

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### ABBREVIATIONS AND SYMBOLS

<p>| 1A | protein of putative proteinase cofactor |
| 1B&lt;sub&gt;Hel&lt;/sub&gt; | putative helicase |
| 1C&lt;sub&gt;Vpg&lt;/sub&gt; | viral protein genome-linked |
| 1D&lt;sub&gt;Pro&lt;/sub&gt; | proteinase |
| 1E&lt;sub&gt;Pol&lt;/sub&gt; | putative RNA-dependent RNA polymerase (RdRp) |
| 2A&lt;sub&gt;HP&lt;/sub&gt; | homing protein |
| 2B&lt;sub&gt;MP&lt;/sub&gt; | movement protein |
| 2C&lt;sub&gt;Cp&lt;/sub&gt; | coat protein |
| ABA | abscisic acid |
| ArMV | <em>Arabis mosaic virus</em> |
| CHS2 | chalcone synthase |
| COX | cytochrome oxidase |
| CP | coat protein |
| ELISA | enzyme-linked immunosorbent assay |
| F3´5´H | flavonoid -3´-5´-hydroxylase |
| F3´H | flavonoid -3´-hydroxylase |
| F3H1 | flavanone-3-hydroxylase 1 |
| F3H2 | flavanone -3-hydroxylase 2 |
| FAO | food and agriculture organisation |
| GCMV | <em>Grapevine chrome mosaic virus</em> |
| GDefV | <em>Grapevine deformation virus</em> |
| GFkV | <em>Grapevine fleck virus</em> |
| GFLV | <em>Grapevine fanleaf virus</em> |
| GLRaV | <em>Grapevine leafroll associated virus</em> |
| GRSPaV | <em>Grapevine rupestris stem pitting virus</em> |
| GTRSV | <em>Grapevine Tunisian ringspot virus</em> |
| GVA | <em>Grapevine virus A</em> |
| GVB | <em>Grapevine virus B</em> |
| Hel | helicase |
| HP | homing protein |</p>
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<td>ICVG</td>
<td>International Council for the study of virus and virus-like diseases of the grapevine</td>
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<tr>
<td>LDOX</td>
<td>Leucoanthocyanidin dioxygenase</td>
</tr>
<tr>
<td>LEA</td>
<td>Late embryogenesis abundant proteins</td>
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<tr>
<td>MP</td>
<td>Movement protein</td>
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<tr>
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1. INTRODUCTION

Grapevines (*Vitis vinifera* L.) are one of the oldest, most widely grown horticultural crops in temperate climates around the world. They currently constitute the most economically important fruit species worldwide. In 2008, the surface cultivated with grapevines was over 7,600,000 ha (FAO, 2011). Grapevines are exposed to several abiotic stresses, and as they are propagated in a vegetative way, they are also exposed to many biotic stresses. These stresses are responsible for dramatic economic losses and represent the main limiting factor for viticulture worldwide. Diseases can cause reduction of grapevine vigour, and often decline of grapevines, which also affects the commercial value and lifetime of a vineyard. Moreover, diseases can cause substantial crop losses and reduced fruit quality (Bovey et al., 1980; Martelli, 1993, 2010, Martelli and Boudon-Padieu, 2006).

One of the most important and widespread virus diseases of grapevine is grapevine fanleaf degeneration (Fuchs et al., 1989; Martelli, 1993), which occurs in all grapevine-growing regions throughout the world (Andret-Link et al., 2004). This disease is caused by *Grapevine fanleaf virus* (GFLV), which is a member of the genus *Nepovirus* (Sanfaçon et al., 2009). GFLV causes degeneration and malformations of leaves, shoots and clusters. It is also responsible for significant economic losses, with the reduction of crop yields by up to 80%, and it affects the longevity of the grapevines (Andret-Link et al., 2004; Mekuria et al., 2009; Raski et al., 1983). Multiple infections by divergent GFLV isolates can occur for a single grapevine (Pompe-Novak et al., 2007; Vigne et al., 2004), as well as mixed infections with other nepoviruses and viruses from different genera (Laimer et al., 2009; Pompe-Novak et al., 2007). GFLV is transmitted by the nematode *Xiphinema index*, by vegetative propagation and grafting, and by distribution of infected vegetative propagation materials (Andret-Link et al., 2004; Mekuria et al., 2009; Raski et al., 1983).

Like other virus diseases, grapevine fanleaf disease is impossible to control in established vineyards. Propagation of uninfected material is one of the most effective ways of controlling the spreading of grapevine fanleaf disease. The grapevine sanitary selection scheme includes the testing of grapevines (nuclear and mother block material) for the presence of GFLV. Different diagnostic methods are available for testing viral infections, which include serological techniques, such as enzyme-linked immunosorbent assays (ELISA), and molecular techniques, such as reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR.
(RT-qPCR) (Čepin et al., 2010). A double antibody sandwich ELISA (DAS-ELISA) is a standard procedure for diagnosis of GFLV (Nolasco, 2003). This assay allows virus detection directly in grapevine extracts, although it lacks the sensitivity required for detecting low virus concentrations; i.e., for latent infections (Rowhani et al., 1992). As these serological techniques lack the sensitivity required for the detection such low virus concentrations, when testing by ELISA, it is essential to choose organs/tissues and times during the season with the highest virus titres, to detect and effectively prevent the spread of the disease.

Symptom expression depends on the host (Vitis species or cultivar) and/or on the virus strain (Legin et al., 1993). No clear association has been observed among different GFLV isolates (according to the genetic variability within RNA2) and expressed symptoms (Pompe-Novak et al., 2007). Host defence responses to GFLV in naturally infected grapevines are also still not completely understood. The signalling pathways and regulatory elements that lead to induction of defence responses have yet to be characterised. Water balance, sugar signalling, and hormone homeostasis have been shown to be involved in resistance to virus infection and drought (Lovisolo et al., 2010; McDowell and Dangl, 2000; Sade et al., 2014), and the complex network of plant hormones (Peleg and Blumwald, 2011) that represents a complex of quantitative trait regulated by numerous genes (Wang et al., 2006) has been shown to regulate plant responses to biotic and abiotic stresses. Therefore, evaluation of the biological role of GFLV in naturally infected grapevines will provide new knowledge about the impact of such virus infection on grapevine physiology and the expression of genes involved in abiotic and biotic stress. Grapevine protection against diseases needs alternative strategies to phytochemicals, which implies the need for thorough knowledge of the innate defence mechanisms. The relationship between host and pathogen and the influence of the pathogen infection on gene expression of the host will provide more efficiency for the breeding of new resistant or tolerant grapevine varieties in the future.
1. Aims

- To investigate a large number of grapevines in several infected vineyards in Slovenia (Karst region, Vipava Valley) and in Italy (Prepotto, in Vigna Petrussa), and to select vineyards and grapevines for analyses.
- To compare the dynamics of GFLV infection and symptoms development in established vineyards and in newly replanted vineyards, where the nematode vector X. index is present.
- To analyse the fluctuation of the virus titre during the season in different parts of the grapevines.
- To analyse how GFLV affects some important agronomical characteristics of the grapevines: such as the number of buds and percentage of bud fertility, the number of clusters, the pruning weight, and the Ravaz index.
- To analyse the grapevine water status, to determine the physiological impact of virus infection on water stress resistance.
- To analyse gene expression under GFLV infection for genes involved in abscisic acid biosynthesis, the anthocyanin and sugar synthesis pathways, plant defence responses, and transcription factors.

2. Hypothesis

- We speculate that we will be able to find grapevines infected only with GFLV (no other viruses) and virus-free grapevines (healthy controls) in selected vineyards in Karst, Vipava Valley and Prepotto.
- We speculate that GFLV infection occurs in newly replanted vineyards where the nematode vector is present within a period of one to two years, and that the symptoms start to be visible the following season.
Infected grapevines show foliar symptoms early in the season, which normally fade during the summer and autumn (Mekuria et al., 2009). We speculate that there will be modifications in the concentrations of GFLV through the season, and also in different plant tissues. We expect that GFLV is not equally distributed in the plant, and we expect high concentrations of the virus in young, fast growing tissues.

GFLV affects some important agronomic characteristics of grapevines, such as yield, and berry and cluster weight (Cigoj, 2015). We speculate that GFLV infection also affects the number of buds, percentage of bud fertility, number of clusters, pruning weight, and Ravaz index.

We speculate that the water status of the grapevines is negatively affected by GFLV infection, and will also be different between the two different grapevine training systems. GFLV infected grapevines are more susceptible to seasonal water deficit.

We speculate that the genes involved in abscisic acid biosynthesis, the anthocyanin and sugar synthesis pathway, plant defence responses, and transcription factors are differentially expressed in GFLV-infected grapevines, compared to healthy grapevines.
2. THEORETICAL BACKGROUND

Vitis vinifera L. is characterised by wide genetic variability, with several thousand varieties cultivated worldwide (Alleweldt et al., 1991). The genetic diversity in grapevines that exists today has been largely determined by their history and by vineyard cultural practices (This et al., 2006). Grapevines are exposed to several abiotic and biotic stress factors. The most common abiotic stresses are drought, high salinity, and high temperature. Biotic stresses are also caused by insects, fungi, bacteria, phytoplasma and viruses.

Grapevines are a host for 63 viruses that have been assigned to 15 families and 28 genera, and other newly identified viruses remain to be classified. The most important viruses are those responsible for, or associated with, grapevine fanleaf disease, leafroll disease, and rugose wood complex (ICVG, 2012; Martelli and Boudon-Padieu, 2006).

Nepoviruses from the family Secoviridae (Sanfaçon et al., 2009) are detrimental for the grape industry from many points of view. They can cause: (a) progressive decline and death of grapevines (Ramdsdell and Myers, 1974); (b) low yields and poor grape quality; (c) shortening of the productive life of a vineyard; (d) low proportions of graft survival; (e) decreased resistance to adverse climatic conditions (Gilmer et al., 1970; Hewitt, 1968); and (f) reduced rooting of propagated material (Stellmach and Bercks, 1963). Most European nepoviruses induce diseases, and these are characterised by a large and relatively confusing array of symptoms (Martelli, 1993).

2.1. The main biological, serological and molecular properties of GFLV

GFLV causes a wide range of symptoms that include reduced vigour and general decline of grapevines (Mekuria et al., 2009). Infected grapevines suffer substantial crop losses, reduced fruit quality, and shortened longevity (Fuchs et al., 1989; Martelli, 1993). Two distinct
syndromes caused by different GFLV strains were described by Martelli (1993). The syndrome of infectious malformations is mainly observed on leaves, shoots and clusters. Here, the leaves are variously and severely malformed, asymmetrically shaped, and have sharply toothed leaf margins. Shoots have abnormal branching, fascination, short internodes, and double nodes, while shoots can develop zig-zag growth. Clusters are smaller and fewer in number, the berries ripen irregularly and are small sized and set poorly. Yellow mosaic syndrome is described as bright chrome-yellow discolorations early in the spring that can affect all vegetative parts (e.g., leaves, shoots, tendrils, inflorescences). The chromatic alterations of the leaves vary from a few scattered yellow spots, which sometimes appear as rings or lines, to extensive mottling of the veins and/or interveinal areas, and to total yellowing. Infected grapevines often occur in patches. Differences in symptomatology caused by GFLV (e.g., bushy-like growth, yellow mosaic symptoms) can also be reflected in terms of the different physiological responses of the grapevines (Martelli, 1993).

*Xiphinema index* is the sole natural vector of GFLV (Brown and Weischer, 1998; Hewitt et al., 1958). The reproduction of this nematode species that belongs to the *Longidoridae* family is parthenogenetic. Its eggs, which are produced in spring, develop into adults through four juvenile stages (Dalmasso and Younes, 1969). Both juvenile and adult *X. index* nematodes can acquire and transmit GFLV to healthy grapevines (Taylor and Raski, 1964). The nematode acquires and transmits GFLV while feeding on root tips of grapevines (Wyss, 2000). A specific and complementary association between *X. index* and GFLV is needed for transmission from grapevine to grapevine (Raski et al., 1983). Interestingly, GFLV is retained by viruliferous individuals for at least 9 months (Taylor and Robertson, 1970). *X. index* can survive and retain GFLV for 4 years in vineyard soil stored at 7 °C to 20 °C in the absence of host plants (Demangeat et al., 2005). A long period of 5 years to 10 years can be required to eliminate this nematode from clay loam soils (Tzortzakakis, 2013). Investigation of the spatial distribution of the *X. index* (Villate et al., 2008) has indicated that nematode patches correlate significantly with those of GFLV-infected grapevines.

Strategies to control GFLV in established vineyards are mainly directed at the eradication or reduction of *X. index* populations, and are based on soil disinfection (Raski et al., 1983; Taylor and Brown, 1997). Nematicides are usually of limited efficacy, especially in heavy and deep soils. In addition, the toxicity of these agrochemicals is acute. Therefore, their use is prohibited in several countries, because of potential adverse environmental effects (Abawi and Widmer,
Prolonged fallow for at least 10 years (Vuittenez et al., 1969) is a very efficient strategy to eradicate *X. index*, but in premium vineyards, this is not acceptable economically.

Natural resistance to GFLV and/or to *X. index* has been identified in a limited number of grapevine species (Walker et al., 1985). The use of resistant rootstock upon which the fruiting cultivars are grafted is often the best way to overcome nematode problems in perennial crops. The new rootstock is known as VR 039-16, and it is a hybrid between *V. vinifera* and *Vitis rotundifolia* that was discovered to be resistant to *X. index* and tolerant to GFLV (Ferris et al., 2012). The concept of pathogen-derived resistance (Sanford and Johnston, 1985) has been applied to engineer resistance against GFLV of grapevines, and several rootstocks that express the *CP* gene of GFLV have been developed (Krastanova et al., 1995; Mauro et al., 1995; Xue et al., 1999). Vigne et al. (2004a, b) showed that transgenic grapevines did not promote the development of viable GFLV recombinants to detectable levels, and did not affect the genetic diversity of the indigenous GFLV populations during a trial period. A lot of work remains to be done by national and international authorities to make scientifically based regulatory decisions for the timely release of such transgenic crops.

Several observations have been reported for the distribution of GFLV in grapevines and for seasonal fluctuations of GFLV titres. GFLV has been detected in buds, young and mature leaves, leaf petioles, phloem of the canes, shoot tips, internodes, roots and flower/ berry clusters (Bouyahia et al., 2003; Fiore et al., 2009; Frantz and Walker, 1995; Rowhani et al., 1992; Walter and Etienne, 1987). Using ELISA, it was successfully shown to be in grapevine leaves throughout the growing season (i.e., from May to October), regardless of whether the leaves were sampled from the bottom or the top of the shoots (Walter and Etienne, 1987). However a gradient of virus titre that increases from the basal leaves towards the apical leaves was reported for the beginning of the growing season, by calculation of the proportion of GFLV-positive samples from apical, medial and basal leaves of GFLV-infected grapevines (Bouyahia et al., 2003). In contrast to the distribution of the virus in leaves, more samples from the basal internodes of GFLV-infected grapevines have tested GFLV positive than from the apical internodes (Bouyahia et al., 2003). In young leaves and shoot tips, ELISA testing showed that the maximal GFLV titre occurs early in the growing season (i.e., May-June) and it drops in July, while it differed between grapevines in August to October (Rowhani et al., 1992). In the phloem, the virus titre was more constant during the growing season, and it was lower outside the growing season (i.e., November to March). As well as in the phloem, outside the growing
season GFLV was also present in roots and sawdust (Rowhani et al., 1992). In two out of three investigated vineyards in California, USA, lower weights of clusters, berries and yields correlated with higher viral titres determined by ELISA, but there was no correlation between virus titre and pruning weights. In addition, negative correlation between viral titre and expression of symptoms was indicated, although it was also implied that the expression of symptoms might be influenced by the grapevine cultivar, the duration of infection, the number of strains of virus that infected the grapevine, and the presence of the nematode in the vineyard (Frantz and Walker, 1995).

GFLV has a bipartite positive-sense RNA genome that is composed of RNA1 and RNA2 (Quacquarelli et al., 1976). Both of these RNA molecules carry a small covalently linked viral protein (VPg) at the 5’-terminus, and a poly (A) tail at the 3’-terminus (Pinck et al., 1991). Each of the RNA molecules encodes a polyprotein that is proteolytically processed to release functional proteins. The RNA1 consists of 7342 nts and codes for a 253-kDa polyprotein that is cleaved by a virally encoded proteinase (also found in RNA1) into five individual proteins, including a protein of unknown function (1A), a putative helicase (1B\textsubscript{Hel}), a genome-linked viral protein, or VPg (1C\textsubscript{VPg}), a proteinase (1D\textsubscript{Pro}), and a putative RNA-dependent RNA polymerase (Rd\textsubscript{RP}) (1E\textsubscript{Pol}) (Margis et al., 1993; Pinck et al., 1991; Ritzenthaler et al., 1991). RNA2 consists of 3774 nts and codes for a polyprotein of 122-kDa that is cleaved by the RNA1-encoded viral proteinase into three individual proteins: a homing protein (2A\textsubscript{HP}) that is necessary for RNA2 replication, a movement protein (2B\textsubscript{MP}), and a coat protein (2C\textsubscript{CP}) (Gaire et al., 1999; R Margis et al., 1993; Ritzenthaler, 1995; Ritzenthaler et al., 1995; Serghini et al., 1990). Analysis of the RNA content of the F13 GFLV isolate revealed the presence of an extra RNA, RNA3, which was found to have the properties of a satellite RNA (Fuchs et al., 1989). RNA3 is dependent on the presence of the two genomic RNAs for its multiplication. The primary structure obtained was 1114 nts in length (Fuchs et al., 1989).

The diversity and the quasi-species nature of the GFLV genome have been assessed in several countries where this virus occurs naturally. Variability studies have been performed using IC-RT-PCR–restriction fragment length polymorphism (RFLP) and sequencing of the complete (Liebenberg et al., 2009; Naraghi-Arani et al., 2001; E. Vigne et al., 2004) and partial (Bashir and Hajizadeh, 2007; Bashir et al., 2007; Fattouch et al., 2005) 2C\textsubscript{CP} gene, the 2B\textsubscript{MP} gene (Bashir et al., 2007), and the complete RNA2 open reading frame (Pompe-Novak et al., 2007). In these studies, for the 2C\textsubscript{CP} gene, nucleotide sequence similarities of 87% and amino acid-
sequence identities of 91% were observed, for the 2B<sup>MP</sup> gene, these were 91% and 93%, respectively, and for the RNA2 open reading frame, they were 93.3% and 97.5%, respectively (Bashir and Hajizadeh, 2007a; Bashir et al., 2007; Fattouch et al., 2005; Naraghi-Arani et al., 2001; Pompe-Novak et al., 2007; Vigne et al., 2004a, 2005). Sequence identities between 2C<sup>CP</sup> gene of different GFLV isolates from South Africa were between 86% and 99% and 94% and 99% at the nucleotide and amino-acid levels, respectively (Liebenberg et al., 2009).

Natural occurrence of recombination in the GFLV 2C<sup>CP</sup> gene was first reported by Vigne et al. (2004 a, b, 2005). Five recombinant isolates were identified among the 347 GFLV isolates investigated; however, no differences were observed between the recombinant and non-recombinant isolates in terms of symptom expression, disease incidence, or grapevine vigour (Vigne et al., 2004a, b; Vigne et al., 2005). Considering the putative roles assigned to the 2C<sup>CP</sup> gene in terms of virus particle structure and stability, virus movement, and interactions with host and vector, limited levels of genetic variation can be tolerated to maintain viability (Andret-Link et al., 2004; Belin et al., 1999).

2.2. The stem water potential and root hydraulic conductivity

Grapevine water status is dependent on climate and soil characteristics (Van Leeuwen et al., 2004) and on grapevine health status (Choi et al., 2013). In drylands such as the Mediterranean region, grapevines are mainly grown under extensive seasonal water deficits. The plant water status indicates the degree of imbalance between water uptake and water loss through transpiration. The rate of water uptake depends on root distribution and soil moisture availability (Smart, 1974; Tesic et al., 2007). Moreover, the soil water supply and grapevine water status are the two main factors that most comprehensively determine berry ripening and their metabolic composition, and consequently wine quality (Chaves et al., 2010; Choné et al., 2001; Cohen et al., 2012; Koundouras et al., 2006; Matthews and Anderson, 1988; Medrano et al., 2003). For grapevines and other fruit crops, fruit quality benefits from a certain level of water deficit, as this facilitates the balance between vegetative and reproductive growth and promotes the flow of carbohydrates to reproductive structures (Chaves et al., 2010; Sivilotti et al., 2005). Water stress has many effects on plant productivity, and it involves reactions at the
intercellular, cellular, and tissue levels. One of the most significant responses is decreased stomatal opening, which enables the plant to alleviate adverse conditions of water status, but concomitantly, this reduces the uptake of CO$_2$ and hence photosynthesis (Smart, 1974; Sampol et al., 2003; Choné et al., 2001).

Virus infections impact on growth and yield parameters, and also decrease the net assimilation rate, stomatal conductance, chlorophyll pigments, and non-photochemical quenching, as has been reported for grapevines infected by GFLV and *Grapevine leafroll associated virus* (GLRaV; Sampol et al., 2003). Endeshaw et al. (2014) reported significantly reduced leaf photosynthesis, stomatal conductance and transpiration before and after visual appearance of GLRaV-3 symptoms. Inhibition of photosynthesis was observed in Chardonnay grapevines infected with GFLV, where they also concluded that the inhibition of photosynthesis itself might be the consequence of elevated levels of reactive free radicals generated against the virus attack (Váradi and Hideg, 2006).

Several physiological indicators are widely used in viticulture to assess plant water status, leaf water potential, root hydraulic conductivity, stomatal conductance, and transpiration (Sivilotti et al., 2005). Following the development of the pressure chamber (Scholander et al., 1965), measurements of water potential ($\Psi$) have been used as a tool to assess the water status of plants (Jones, 1990; Koide et al., 1989). This has been correlated with various aspects of grapevine physiology (Naor et al., 1994), vegetative growth (Schultz and Matthews, 1988, 1993), and reproductive growth and yield (Greenspan et al., 1996; Grimes and Williams, 1990). Leaf water potential is a standard indicator of plant water status, but predawn and stem water potential are considered better indicators in grapevines (Choné et al., 2001b). The stem water potential ($\Psi_{STEM}$) has been shown to be less variable than the leaf water potential ($\Psi$) and has improved the ability to detect small, but statistically significant, differences among treatments (McCutchan and Shackel, 1992). In addition, $\Psi_{STEM}$ has been shown to be a linear function of the water applied (Lampinen et al., 1995) and the soil water availability (Stevens et al., 1995). Grapevine water status can be assessed accurately by means of $\Psi_{STEM}$ or carbon isotope discrimination measured for the grape sugars (Leeuwen et al., 2009). Grapevines without any water stress show midday $\Psi_{STEM}$ values above −0.6 MPa, with weak water restriction reflected in values from −0.6 MPa to −0.9 MPa. Then $\Psi_{STEM}$ values from −0.9 MPa to −1.1 MPa correspond to moderate water deficit. Moderate to severe water deficit is reflected in $\Psi_{STEM}$
values from $-1.1$ MPa to $-1.4$ MPa, while $\Psi_{STEM}$ values lower than $-1.4$ MPa correspond to severe water stress (Leeuwen et al., 2009).

The root hydraulic conductivity (RHC; nL H$_2$O s$^{-1}$ m$^{-1}$ MPa$^{-1}$) is a parameter that describes modifications in water uptake from the root–soil interface to the apoplast and guard cells in the leaves (Schultz, 2003). The xylem tissue development, the hydraulic system, and thereby the whole-plant hydraulic conductance is influenced also by genetics (Tramontini, 2012). The primary consequence of a root-to-shoot hydraulic signal is generally increased abscisic acid (ABA) biosynthesis in the shoot, which regulates the stomata (Christmann et al., 2007) and leaf growth (Chazen et al., 1995; Neumann et al., 1997). Increases in ABA are not only dependent upon increases in ABA biosynthesis, but are also influenced by ABA redistribution and transport (Wilkinson and Davies 2002). ABA promotes water uptake and cell-to-cell water flow by inducing the aquaporin genes (Hose et al., 2000). Therefore, the aquaporins (AQPs; as membrane channels; Tyerman et al., 2004; Kaldenhoff et al., 2007; Maurel et al., 2008) have important roles in the control of the water movement. The AQPs are controlled by the cellular water balance and transpiration rate, with subsequent effects on hormonal balance and sugar signalling. Moreover, tonoplast-intrinsic proteins (TIPs), which belong to the AQP superfamily, have major roles in cell water balance (Maurel et al., 2008; Reuscher et al., 2013). Plants expressing the TIP-AQP gene were shown to be drought and salt tolerant. Interestingly, Sade et al. (2014) showed that the TIP-AQPs are involved in resistance of tomato to Tomato yellow leaf curl virus. In these virus-infected plants, a reduction in stomatal opening and lowered transpiration rate were correlated with water retention (Hall and Loomis, 1972; Lindsey and Gudauskas, 1975; Keller et al., 1989). Data from the recent studies have shown that plants infected with four different RNA viruses, Brome mosaic virus, Cucumber mosaic virus, Tobacco mosaic virus and Tobacco rattle virus, show better tolerance and survival to drought or cold stress (Xu et al., 2008). Different combinations of applied stress, including drought, heat stress and virus infection, in Arabidopsis thaliana enhanced the expression of different sets of genes (Prasch and Sonnewald, 2013). Abiotic stress signalling has the potential to override biotic stress signalling in situations of simultaneous stress.

Plants that show a conservative strategy and those with a less conservative strategy for the management of their water balance are referred to as isohydric and anisohydric, respectively. Studies have shown that an interaction between hydraulic properties and ABA might be required to cause stomatal closure in isohydric species, whereas stomatal control in anisohydric
species is strictly under hormonal control (Tardieu et al., 1996). Lovisolo et al. (2010) assigned *Vitis* genotypes to isohydric or anisohydric in response to water stress. Higher levels of xylem ABA correlated to better stomatal control. Grapevines with anisohydric behaviour have less stomatal control, and varieties with tight stomatal control have isohydric characteristics (Schultz, 2003). It has also been shown that the differences in the responses of varieties are linked to differences in their ABA content.

Plant responses to the environment are regulated via networks that are formed as intricate signalling pathways. Like other drought-tolerant plants, grapevines have developed unique strategies to cope with and adapt to abiotic and biotic stresses. Also, a high degree of phenotypic plasticity is required to balance plant responses to different stress combinations at different developmental stages and for varying durations. The phenotypic plasticity is mainly determined by the plant genome. In parallel, biotic agents like viruses are involved in the manipulation of the plant genome in a specific way (Lalić et al., 2011).
Figure 1: Schematic view of water-stress effects on grapevine leaves, berries, shoots and roots (after Lovisolo et al., 2010). Summary of the water stress effects on grapevine leaves, berries, shoots and roots. Leaves: Different signals contribute to stomatal closure and a decrease in photosynthesis. Down-regulation of aquaporins (AQPs) can decrease mesophyll conductance to CO₂. The accumulation of soluble sugars results in inhibition of photosynthesis. In isohydric varieties, the leaf water potential is kept above a certain threshold by co-regulation of stomatal conductance (gₛ) and leaf hydraulic conductivity, to avoid cavitation. In anisohydric varieties, the leaf water potential decreases along with the osmotic adjustment and changes in cell-wall elasticity. Berries: Berry quality and the biosynthesis and accumulation of polyphenols are affected in both isohydric and anisohydric varieties. Phloem influx is accompanied by a decrease in cell turgor and berry volume. Especially when water stress occurs pre-veraison, it induces an increase in sugar influx and ABA, which can influence key steps of the phenylpropanoid biosynthetic pathway and can trigger a defence response upon water stress. Shoots: Water stress inhibits internode extension, leaf expansion, and elongation of tendrils, and therefore limits transpiration. Decreases in the mean diameter of the xylem vessels lower xylem hydraulic conductivity and can prevent embolism, as smaller vessels are less susceptible to cavitation. APQs can assist in embolism repair. The apoplast is alkalised and the ABA pool is enriched in the low permeability conjugated form of ABA-GE, a long-distance stress signal. Cytokinin concentrations can fall by 50%, and this accounts for growth inhibition. Auxins can regulate cytokinin biosynthesis and also xylem vessel size. Roots: Roots grow into deeper, wetter soil layers. PRP genes have a role in the initiation of new roots on grape stem cuttings, probably by altering the cell-wall mechanical properties.
properties, to enable root emergence. Increased suberisation of the exodermis and/or endodermis increases the hydraulic resistance along the apoplastic pathway. Root symplastic water transport is improved by osmotic adjustment and by increased activity of AQP. Anisohydric behaviour: increased expression of AQP avoids a major reduction of $K_h$ and so avoids vulnerability to embolism. Isohydric behaviour: no change in AQP transcript levels, which combined with increased suberisation, leads to a lower $K_h$; however, tight control on stomatal regulation avoids excessively low xylem water potentials, and therefore minimises the risk of cavitation.

Every aspect of plant responses to biotic and abiotic stresses is regulated by plant hormones (Peleg and Blumwald, 2011). ABA is the main drought-induced hormone, and it regulates the expression of many genes related to drought responses (Iuchi et al. 2001; Hao et al. 2009; Su et al. 2011). Moreover, the ABA-inducible target gene has been defined where its expression is important for the establishment of abiotic and biotic stress tolerance (Mauch-Mani and Mauch, 2005). Advances in ABA research have included the identification of ABA-responsive elements, as reviewed in Guo et al., (2011), and the identification of the ABA signal transduction pathway in V. vinifera (Boneh et al., 2012). Furthermore, it has been shown that exogenous ABA induces expression of the $CBF/DREB1$ genes in Vitis spp. (Xiao et al., 2006). Also, the identification of multiple plasma-membrane ABA transporters (Boursiac et al., 2013; Kang et al., 2010; Kuromori and Shinozaki, 2010; Umezawa et al., 2010), new insights into the ABA connection with the circadian clock signalling pathway (Castells et al., 2010; Seung et al., 2012), and examination of the ABA role in localising reactive oxygen species in guard cells for stomatal closure (Hashida et al., 2010; Zhao et al., 2010) have all raised new aspects in ABA research (Kline et al., 2010). Decreases in water content and turgor are likely to be required to trigger the accumulation of ABA (Creelman and Zeevaart, 1985; Pierce and Rashke, 1980), which then causes stomatal closure, to prevent a further decrease in water content (Rogiers et al., 2012).
2.3. Genes involved in abiotic and biotic stress responses

ABA biosynthesis is largely induced through transcriptional regulation of the ABA-biosynthetic genes. Those regulators are induced by distinct agents. Among these agents, the most widely studied are the abiotic agents (e.g., drought, salt stresses). On the other hand, biotic stresses have been more intensively investigated in recent years (Westwood et al., 2013). The ABA biosynthetic pathway is regulated by the key enzyme NCED (Wan et al., 2009). A positive correlation was shown between increased transcription of NCED mRNA and increased levels of the NCED protein during the course of water deficit stress. Furthermore, ABA levels increased due to the overexpression of the NCED gene (Qin and Zeevaart, 2002). Several observations support the idea that ABA can stimulate its own degradation, but not its own production. In tomato plants, it has been shown that the NCED gene is not induced by exogenous ABA (Thompson et al., 2000), and in cowpea, ABA was not able to activate the NCED genes (Iuchi et al., 2000). In the past decade, it was suggested that an initial induction of ABA biosynthesis rapidly stimulates further biosynthesis of ABA, through a positive feedback loop (Xiong and Zhu, 2003).

Adaptation to abiotic and biotic stress is often regulated by the combined activity of interconnected ABA-dependent and ABA-independent signalling pathways. Dehydration tolerance mechanisms, such as accumulation of RD22 and other drought-induced proteins (i.e., the LEA protein) can be initiated before significant dehydration occurs, as a way of preparing the plant for further decreases in water content. Under dehydration and ABA-inducible expression of the RD22 gene, the MYC/MYB transcription factors function as transcriptional activators. The interaction of the MYC and MYB proteins in ABA-induced gene expression in vegetative tissues under dehydration stress conditions has been shown (Abe et al., 1997). There also appears to be a link between genes that are induced under the control of MYC-like and MYB-like regulatory genes and those induced during anthocyanin biosynthesis, which are discussed later.

The WRKY proteins comprise one of the largest families of transcription factors in plants (Rushton et al., 2012). Structural and expression analyses show that the WRKY proteins are a class of important regulators of growth and development and that they have key roles in
responses to stresses. Therefore, various physiological and molecular processes in plants are known to be induced or repressed by WRKY transcription factors. The WRKY proteins have key roles in the regulation of pathogen-induced defence responses (Yang et al., 2013). Westwood et al., (2013) reported that Cucumber mosaic virus infection induced drought tolerance through the ABA-mediated signalling pathway in A. thaliana. Furthermore, WRKY transcription factors have been shown to be involved in abiotic stress responses (Fowler and Thomashow 2002; Seki et al. 2002; Mare et al. 2004). They are also involved in various physiological processes (Eulgem and Somssich, 2007; Eulgem, 2006), and positive and negative regulation of the WRKY promoters by specific WRKY proteins has been observed. Also, many of these stress responses are regulated by ABA. New data demonstrate that the WRKY transcription factors are key nodes in ABA-responsive signalling networks (Rushton et al., 2012). In Arabidopsis, several genes that encode WRKY transcription factors have been shown to be expressed during drought stress and are involved in ABA signalling networks. The expression patterns of the WRKY genes have revealed their important roles in several functions in developmental stages of vegetative and reproductive growth and stress responses. Furthermore, WRKY23 is needed for correct root growth and development, through stimulation of the local biosynthesis of the flavonols (Grunewald et al., 2013, 2011).

Under abiotic and biotic stress, sugar metabolism is very important in the modulation of plant development, as sugars are involved in signal transduction pathways (Rolland and Sheen, 2005). Sugars have important roles during plant growth and development under abiotic stresses through the regulation of carbohydrate metabolism. Sucrose synthase (SuSy) is a key enzyme in sucrose metabolism, and it catalyses the reversible conversion of sucrose and UDP to UDP-glucose and fructose (Gupta and Kaur, 2005). In various plant species, SuSy has a role in supplying substrates for starch and cell-wall synthesis, including supplying energy to companion cells for phloem loading. The SuSy gene has also been reported to be up-regulated under water stress (Ho et al., 2001).

On the other side to the adaption to biotic stresses, specific molecular mechanisms have developed in plants to detect pathogens and pests and to activate the defence responses. Protein recognition receptors are innate in the plant immune system, which is dependent on the specific detection by the plant of conserved molecules of the pathogen, which are called pathogen-associated molecular patterns (Denancé et al., 2013). In parallel, it has been shown that pathogens have developed sophisticated molecular mechanisms to deregulate the biosynthesis
of plant hormones and/or to interfere with plant hormonal signalling pathways, thus facilitating the overcoming of plant defence mechanisms (Dangl and Jones, 2001; Dodds and Rathjen, 2010; Jones and Dangl, 2006; Koornneef and Pieterse, 2008). Therefore, fine-tuned regulation of these immune responses is necessary, because phytohormone pathways are connected to each other in a complex and obscure network in which all of them contribute to the hormonal balance. For example, in the signalling backbone of the salicylic acid and jasmonic acid pathways, ABA, auxin, cytokinins, ethylene and gibberellins are all considered to be hormonal modulators (Koornneef and Pieterse, 2008; Pieterse et al., 2012; Verhage et al., 2010). Salicylic acid, ethylene and jasmonic-acid-mediated signalling pathways have essential roles in resistance to pathogens (Gimenez-Ibanez and Solano, 2013; Robert-Seilaniantz et al., 2011; Thaler et al., 2012). Salicylic acid signalling positively regulates plant defences against biotrophic pathogens, whereas the ethylene/ jasmonic-acid pathways are commonly required for resistance to necrotrophic pathogens, as reviewed in Glazebrook (2005).

One of the known defence mechanisms against viral pathogens in plants is hormone-mediated resistance, which is mediated by salicylic acid and methyl-salicylate in systemic acquired resistance. Methyl salicylate is synthesised in plants from salicylic acid by methyltransferases, and it has a role in the defence against microbial and insect pests. Methyl salicylate travels through the phloem as a systemic acquired resistance signal to the distal tissue (Nicaise, 2014). Recent advances in the understanding of the pathways and genes controlling the synthesis of salicylic acid have shown that salicylic acid can undergo a number of biologically relevant chemical modifications, including glucosylation, methylation, and amino-acid conjugation. Most modifications render salicylic acid inactive, while at the same time they allow fine-tuning of its accumulation, function, and/or mobility (Dempsey et al., 2011). Methylation inactivates salicylic acid while increasing its membrane permeability and its volatility, and this thus allows more effective long-distance transport of this defence signal. Methyl salicylic acid is synthesised from salicylic acid by the enzyme S-adenosyl-L-methionine salicylic acid carboxyl methyltransferase (O-methyltransferase, SAMT) (Ament et al., 2010; Tieman et al., 2010). The salicylic acid signal transduction pathway leads to the expression of the PR gene OLP. Osmotin or osmotin-like protein, is a PR-5 family member, and it is differentially induced in plants by abiotic and biotic stresses.

Water uptake conditions influence the grapevine phenology and grape ripening. Mild water deficit enhances grape quality for the production of red wines (Tregoat et al., 2002), as an
impact of lower midday $\Psi_{STEM}$ was shown for berry quality (e.g., anthocyanin and phenol content) and weight. Severe water stress is detrimental to grape quality, as the extractability of polyphenols can also be reduced dramatically (Sivilotti et al., 2005). Quality losses through severe water stress can be avoided through the use of drought-adapted plant material, appropriate canopy management, yield reduction, or implementation of irrigation (Leeuwen et al., 2009).

Plant secondary metabolites, such as the flavonoids, can have important effects on plant physiology and can protect plants against various biotic and abiotic stresses. There are three major classes of flavonoids (i.e., anthocyanins, proanthocyanidins, flavonols) and these are synthesised via the flavonoid biosynthetic pathway. Their antioxidant capacity is well known. Indeed, increased osmoprotectant and antioxidant levels in infected plants correlate with improved plant tolerance to abiotic stress (Xu et al., 2008).

Abiotic stresses, such as drought and extremes of light exposure and temperature, can magnify the production of anthocyanins in grapevines. Anthocyanins represent the majority of the fast-rate antioxidants (Pérez-López et al., 2014). Overproduction of reactive oxygen species in the cells results in the intervention of the antioxidant systems of the grapevine leaves with infection by GFLV (Sgherri et al., 2013). The anthocyanin contents as individual and total anthocyanins in berries are increased by virus infection in both cultivars and training systems. The impact of virus infection was observed on anthocyanin content of berries of cultivars ‘Schioppettino’ and ‘Refošk’. In the cultivar ‘Schioppettino’ trained using a single Guyot training system, studies of anthocyanin content have been complemented also by studies of the expression of genes involved in the flavonoid biosynthetic pathway. The greatest impact of GFLV on gene expression was observed in the berry skin. The GFLV infection affected the expression of genes involved in anthocyanin biosynthesis. Under the influence of GFLV infection, the biosynthesis of 3’-5’-hydroxylated anthocyanins (e.g., delphinidin, petunidin, malvidin) was increased due to up-regulation of $F3H1$. Under the influence of GFLV infection, the ratio between tri-substituted and di-substituted anthocyanins increased due to up-regulation of F3´5´$H$ and down-regulation of F3´$H$ (Cigoj, 2015).

The induction of anthocyanin synthesis is triggered by regulatory genes. The enhanced expression of $MYB$ regulatory genes in water-stressed berries suggested a coordinated up-regulation of structural genes in the general flavonoid pathway and anthocyanin-specific genes
(Castellarin et al., 2007). Moreover, the MYB gene was induced in GLRaV-3–infected leaves (Gutha et al., 2010a), and was up-regulated by Pseudomonas syringae infection in Arabidopsis (Stracke et al., 2001).

The key steps of the flavonoid pathway for anthocyanin biosynthesis are divided into phases: colourless precursors, coloured but unstable pigments, and stable pigments (Castellarin et al., 2007a). The first step in anthocyanin biosynthesis is regulated by the chalcone synthase (CHS) genes, a small family of CHSs is needed to recruit flavonoid precursors from the phenylpropanoid pathway (Castellarin et al., 2007b). Expression of CHS2 has been implicated in the production of proanthocyanidins in unpigmented tissues of grape cultivars, in leaves, and in unripe berries. Flavanone 3-hydroxylase (F3H) catalyses the stereospecific 3β-hydroxylation of (2S)-flavanones to dihydroflavonols. Proanthocyanidins are important quality components of many plants and they contribute to health benefits and fruit taste. During proanthocyanidin synthesis in grapes, the genes that encode both leucoanthocyanidin reductase (LAR) and anthocyanidin reductase are expressed and regulated in temporal-specific and tissue-specific manners (Bogs et al., 2005). Leaves also contain significant levels of proanthocyanidins, although the composition differs from that in berry skins, and the expression of LAR2 is low in the first four stages of leaf development and increases in fully expanded leaves (Bogs et al., 2005). LAR converts leucocyanidin to catechin, and then an epimerase converting catechin to epicatechin (Stafford, 1990). It has been shown that leucoanthocyanidin dioxygenase (LDOX) is essential for proanthocyanidin synthesis. In Arabidopsis, anthocyanidin reductase uses cyanidin as a substrate, rather than leucocyanidin (Figure 2).
Grapevines are generally well adapted to arid and semi-arid climates, and they appear to primarily rely on drought avoidance mechanisms under water-stress situations (Chaves et al., 2010). Indeed, plants have evolved highly complex regulation of root-to-shoot growth to achieve maximum fitness with the available resources under abiotic and biotic stress. In the present study, we address the modulation of the water status and the expression of genes involved in drought responses and virus infection. Some studies have investigated the impact of water deficit on grapevines at the physiological and molecular levels but to the best of our knowledge, none of those have studied the impact of GFLV and water deficit on grapevine at the same time.
3. EXPERIMENTAL

3.1. Selection of vineyards and relevant data

This study was carried out based on 11 vineyards in Slovenia and Italy. Table 1 gives the parameters of the selected vineyards, such as their locations, the cultivars, and the research focus.

Table 1: Description of selected vineyards. SWP, stem water potential.

<table>
<thead>
<tr>
<th>Location</th>
<th>Owner</th>
<th>Vineyard area (ha)</th>
<th>Altitude (m a.s.l.)</th>
<th>Cultivar</th>
<th>Training system:</th>
<th>Year established</th>
<th>Research focus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepotto</td>
<td>Vigna Petruzza</td>
<td>~2</td>
<td>150</td>
<td>‘Schioppettino’</td>
<td>Single Guyot</td>
<td>2000 (single Guyot) and 1983 (double Guyot)</td>
<td>SWP and gene expression analysis</td>
</tr>
<tr>
<td>Komen</td>
<td>Vinakras z.o.o</td>
<td>~4</td>
<td>280</td>
<td>‘Refošk’</td>
<td>Single Guyot</td>
<td>1996</td>
<td>SWP and gene expression analysis</td>
</tr>
<tr>
<td>Krajna Vas</td>
<td>private</td>
<td>~0.1</td>
<td>270</td>
<td>‘Refošk’</td>
<td>Sylvoz</td>
<td>Before 1930</td>
<td>Spatiotemporal distribution of GFLV</td>
</tr>
<tr>
<td>Tomaj</td>
<td>private</td>
<td>~2</td>
<td>362</td>
<td>‘Refošk’</td>
<td>Pergola</td>
<td>Before 1970</td>
<td>Symptom observation</td>
</tr>
<tr>
<td>Dutovlje</td>
<td>private</td>
<td>~2</td>
<td>313</td>
<td>‘Refošk’</td>
<td>Pergola</td>
<td>Before 1970</td>
<td>Spatiotemporal distribution of GFLV</td>
</tr>
<tr>
<td>Dutovlje</td>
<td>private</td>
<td>~0.1</td>
<td>313</td>
<td>‘Refošk’</td>
<td>Guyot</td>
<td>Replanted in 2010</td>
<td>Dynamics of GFLV</td>
</tr>
<tr>
<td>Vrhpolje</td>
<td>private</td>
<td></td>
<td>137</td>
<td>‘Župlanka’</td>
<td>Casarsa</td>
<td>Before 1970</td>
<td>Spatiotemporal distribution of GFLV</td>
</tr>
<tr>
<td>Vrhpolje</td>
<td>private</td>
<td>~2</td>
<td>137</td>
<td>‘Laški rizling’</td>
<td>Casarsa</td>
<td>Before 1970</td>
<td>Dynamics of GFLV</td>
</tr>
<tr>
<td>Lože</td>
<td>BF Ljubljana</td>
<td>~1</td>
<td>139</td>
<td>‘Volovnik’</td>
<td>Sylvoz</td>
<td>1985</td>
<td>Spatiotemporal distribution of GFLV</td>
</tr>
<tr>
<td>Kromberk</td>
<td>BF Ljubljana</td>
<td>~1</td>
<td>135</td>
<td>‘Malvazija’</td>
<td>Sylvoz</td>
<td>1930-1940</td>
<td>Spatiotemporal distribution of GFLV</td>
</tr>
</tbody>
</table>
3.2. Selection of cultivars

Cultivar ‘Refošk’ represents one of the oldest and most frequently cultivated varieties in the Karst region. It is the fourth-most-frequent cultivar in Slovenia, and has great economic importance. ‘Refošk’ grapes grown in the Kras district are used to produce the highly appreciated wine known as PTP Teran, which is protected with a recognised traditional denomination (Rules on wine, 2013).

Cultivar ‘Schioppettino’ was derived from the Friuli-Venezia-Giulia region, from the area between Prepotto and Goriška brda. The historical references to ‘Schioppettino’ date from 1282. In 1863, ‘Schioppettino’ was described in the wine grape catalogue for Friuli-Venezia-Giulia. Like other old varieties, in the early 20th century, ‘Schioppettino’ was also replaced with varieties from France, and as a consequence at that time, it was almost lost. In the wine-growing region of Primorska, this cultivar is known as ‘Pokalca’, and it is classified as a permitted cultivar (Rules on the demarcation, 2003).

Cultivar ‘Volovnik’ is an autochthonous cultivar that is mainly planted in Vipava Valley. The long viticulture tradition in Vipava Valley has maintained some of the local grapevine cultivars such as ‘Volovnik’. ‘Volovnik’ was first described by Matija Vertovec in the book “Vinoreja” in 1844 as a well-known, but rarely planted, cultivar. The synonym of ‘Volovnik’ is ‘Drenik’ (Vertovec, 1844). There is no ampelographic description of ‘Volovnik’ in any of the literature.

Cultivar ‘Laški Rizling’ is also known as ‘Welschriesling’, ‘Olasz Riesling’ and ‘Graševina’, and it is a white wine grape variety. Indeed, it is the most widely cultured variety in Slovenia for the production of white wine.

Cultivar ‘Malvasia’ is also known as ‘Malvazia’, and it is a group of wine grape varieties that have been grown historically in the Mediterranean region, the Balearic Islands, the Canary Islands and the island of Madeira, and it is now grown in many of the winemaking regions of the world. In the past, the names ‘Malvasia’, ‘Malvazia’, and ‘Malmsey’ were used interchangeably for Malvasia-based wines; however, in modern oenology, ‘Malmsey’ is now used almost exclusively for a sweet variety of Madeira wine made from the ‘Malvasia’ grape.

Cultivar ‘Župlanka’ is an old local variety, and it is a very rare cultivar in the Vipava Valley.
In this study, ‘Refošk’ was trained according to the single Guyot training system, while ‘Schioppettino’ was trained in both single and double Guyot training systems. For ‘Schioppettino’, both of the training systems were selected due to the different ratios between canopy and yield.

### 3.3. Plant material

To analyse the presence of virus infection, samples were collected from individual grapevines in vineyards in the Slovenian Karst region (vineyards in Dutovlje, Tomaj and Komen), in the Slovenian Vipava Valley (Lože), and in Italy (Prepotto).

For two vineyards in two different locations, the grapevines were selected to monitor the dynamics of GFLV spread throughout the vineyard, and to monitor the time needed for transmission of GFLV by *X. index* to young grapevines, and the time needed for symptoms to develop. Grapevines of ‘Laški rizling’ in a vineyard in Vrhpolje, and newly replanted grapevines of ‘Refošk’ in a vineyard in Dutovlje were sampled to be tested individually for the presence of GFLV by DAS-ELISA. The presence of *X. index* in the soil was analysed in previous studies in framework of the L4-0165 Research Programme. In the vineyard in Vrhpolje for 2011, leaf samples were collected from 30 grapevines of ‘Laški rizling’. Then for 2013, leaf samples were collected from 32 symptomatic and asymptomatic grapevines. In the vineyard in Dutovlje in 2011, leaf samples were collected from 10 grapevines of ‘Refošk’. Then in 2013, leaf samples were collected from 26 symptomatic and asymptomatic grapevines of ‘Refošk’.

To study the distribution of GFLV in the grapevines and the fluctuation of the GFLV titre during the season, samples of young and mature leaves, flower/berry clusters, tendrils, roots and phloem were collected monthly during the vegetative period from May to September. Samples of roots and phloem were collected also out of the vegetative period, in January. Samples were taken in three subsequent years (from January 2008 until September 2010) from six grapevines (*V. vinifera* L.) of two cultivars (‘Refošk’, ‘Volovnik’) in three different vineyards in the Slovenian Karst and Vipava Valley regions, as: grapevine 1: Refošk DU 3/13; grapevine 2:
Refošk 26 6/2; grapevine 3: Volovnik 2/55; grapevine 4: Refošk 26 6/4; grapevine 5: Volovnik 2/52; grapevine 6: Refošk DU 2/19.

To study the variability of the GFLV titre in the grapevines, additional samples were taken from five of the six above-mentioned grapevines (grapevines 1-5) in 2010. Samples of young and mature leaves, tendrils and phloem were collected separately from basal, medium and apical parts of the canes. For the phloem, three separate samples were taken from each of the three parts of canes (basal, medium and apical).

To study the distribution of GFLV in berries, three not yet coloured (i.e., green) and three already coloured (i.e., red) berries were randomly collected from three grapevines of ‘Refošk’ (grapevine 1, grapevine 2, grapevine 4) in August 2010. For analyses, each berry was divided into three parts: skin, flesh and seeds.

To compare the GFLV titres in the different grapevine cultivars, young shoots (i.e., young leaves, shoot tips, tendrils) from 54 grapevines of ‘Refošk’ (23 grapevines), ‘Volovnik’ (10), ‘Malvazija’ (8), ‘Laški rizling’ (11) and ‘Župlanka’ (2) were sampled monthly during the vegetative period (from June to September 2008) at six different locations in the Slovenian Karst region (Dutovje, Komen, Krajna Vas) and the Vipava Valley (Lože, Vrhploje, Kromberk). Also, from 30 of these 54 grapevines of ‘Refošk’, ‘Volovnik’ and ‘Malvazija’, phloem scrapings were sampled out of the vegetative period, in January 2009.

Healthy (i.e., grapevines that were healthy in the following year were defined as uninfected grapevines for the tested viruses) and GFLV-infected grapevines were selected, to evaluate the grapevine water status and GFLV effects on the important agronomical characteristics of the grapevines (i.e., measurements of number of clusters, buds/ blind buds, and shoots, pruning weight, internode length, Ravaz index). In the vineyard in Prepotto, 43 grapevines were selected, and with the vineyard in Komen, where 22 grapevines were selected. At the end of June or the beginning of July, the numbers of clusters, buds and shoots were determined. In winter of 2011, the pruning weights and internode lengths were measured.

In the vineyard in Komen in 2011, the stem water potential (SWP) of 64 healthy and 8 GFLV-infected grapevines was measured, and in 2012, the SWP of 7 healthy and 8 GFLV-infected grapevines of cultivar ‘Refošk’ was measured. In the vineyard in Prepotto, the SWP of 21 healthy and 24 GFLV-infected grapevines of cultivar ‘Schioppettino’ was measured. Here, 12
healthy and 13 GFLV-infected grapevines were trained using the single Guyot training system, and 9 healthy and 11 GFLV-infected grapevines were trained using the double Guyot training system.

For the gene expression analyses, leaf samples from 21 healthy and 24 GFLV-infected grapevines of ‘Scioppettino’, and from 15 grapevines of ‘Refošk’, were collected four times in year 2012, in the vineyards in Prepotto and Komen. Leaves of the size of the hand were collected from the tops of the grapevines, and were immediately frozen in liquid nitrogen in 50-mL plastic tubes (Falcon). The samples were stored at -80 °C until analysis.

For the pot trial to study the impact of GFLV on the root hydraulic conductivity (RHC) and the water status of grapevines under the controlled conditions of the greenhouse, in January 2012, 1-year-old shoots of ‘Schioppettino’ were collected in the vineyard in Prepotto from six healthy and seven GFLV-infected grapevines. These samples were stored at 4 °C until they were propagated in the greenhouse. In April, self-rooted grapevines were planted into 3-L pots. From June 26, the water stress condition was set up for two-thirds of the grapevines for each treatment.

In the pot trial, the SWP was measured in individual plants every 3 days after applying water deficit for each treatment. To measure this, nine healthy and nine GFLV-infected grapevines were sampled at all of the time points after the measurements of RHC and SWP. The fresh and dry weights of the roots and shoots were measured. To analyse gene expression in the grapevines planted in pots, the third leaves from the top were collected from nine uninfected grapevines and nine GFLV-infected grapevines for each treatment and at all of the time points; these were immediately frozen in liquid nitrogen in tubes. For each time that the SWP measurements were carried out, the leaves from the tips of the grapevines were collected and stored to -80 °C for further gene expression analyses.
3.4. Recording of GFLV symptoms

For grapevines infected only with GFLV (i.e., that tested negative to all of the other investigated viruses) of six different cultivars (‘Schioppettino’, ‘Refošk’, ‘Laški rizling’, ‘Volovnik’ ‘Malvazija’), the symptoms on the leaves, shoots and flower/ berry clusters were recorded through the whole season for three sequential years. The number of different symptoms were counted for each grapevine, in terms of the 20 recorded symptoms of: fan leaves, asymmetrical leaves, sharply toothed leaf margins, crinkled leaves, smaller leaves, spot chlorosis, chlorotic mottling, yellow mosaic, shoot bifurcations, bifurcation on internodes, bifurcation on nodes, short internodes, double nodes, shoots growing together, flattened stems, zig-zag growth, dense leaf growth, bushy like growth, shortened shoots, and yield reduction.

3.5. Recording agronomical characteristics of the grapevines

At the end of June or the beginning of July, the number of clusters, buds, blind buds and shoots per grapevine were recorded. In winter, the pruning weights of the individual grapevines were recorded. The lengths of the individual grapevine shoots were measured and the numbers of nodes were counted. From the numbers of buds and shoots, the numbers of the blind buds were calculated. The average lengths of the internodes were calculated by rating the lengths of the shoots with the number of nodes, and considering the numbers of nodes reduced by one. The mean Ravaz indices were calculated as the ratio between the yield weight and the pruning weight of the healthy and GFLV-infected grapevines.
3.6. Measurements of water status of grapevines in vineyards

The grapevine water status was monitored in the two sequential vegetative seasons of 2011 and 2012 (Table 2). Measurements were taken at least four times per season, once for each of the following key phenological stages: BBCH 53 (inflorescences clearly visible); BBCH 77 (berries beginning to touch); BBCH 81 (beginning of ripening: berries beginning to develop their variety-specific colour); and BBCH 89 (berries ripe for harvest). These included measurements of stem water potential ($\Psi_{\text{STEM}}$) with a Scholander pressure chamber (Table 2). Two leaves on each of 60 selected grapevines were covered with aluminium-foil-coated plastic bags at midday for at least 1 h, to allow the stem and leaf water potential to equilibrate (Choné et al., 2001). Then, the leaves were removed and $\Psi_{\text{STEM}}$ was measured according to Scholander et al. (1965) (Figures 3, 4). A maximum of 30 s elapsed between the cutting of the leaves and the measurements.

Table 2: Schematic representation of the BBCH phenophases, time points, cultivars (‘Schioppettino’, ‘Refošk’) and time scale of the SWP measurements in 2011 and 2012 and the sampling strategy for gene analyses in the vineyards in 2012.

<table>
<thead>
<tr>
<th>BBCH phase</th>
<th>inflorescences clearly visible</th>
<th>berries beginning to touch</th>
<th>beginning of ripening: berries begin to develop variety-specific colour</th>
<th>berries ripe for harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME POINTS in 2011</td>
<td>BBCH 53</td>
<td>BBCH 77</td>
<td>BBCH 81</td>
<td>BBCH 89</td>
</tr>
<tr>
<td><strong>MEASUREMENTS OF SWP</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TIME POINTS in 2012</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td><strong>MEASUREMENTS OF SWP</strong></td>
<td></td>
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<tr>
<td><strong>SAMPLING FOR GENE ANALYSES</strong></td>
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</tr>
</tbody>
</table>
Figure 3: (A) Taking the measurements of the stem water potential (SWP) in a vineyard. (B) The Scholander pressure chamber in a vineyard.

Figure 4: Leaves covered with aluminium-covered plastic bags in a vineyard, for measurements of the stem water potential (SWP).
3.7. Measurement of the grapevine water status and the root hydraulic conductivity in potted plants

- Measurement of stem water potential

To measure the SWP, one leaf of six plants for each treatment was covered with aluminium-foil-covered plastic bags 1 h before the measurement. The measurements of the plant water status of healthy and infected grapevines were carried out using a Scholander pressure chamber.

Control plants were irrigated according to their water status and kept at a $\Psi_{STEM}$ between -0.2 MPa and -0.6 MPa. Using the midday $\Psi_{STEM}$ as the grapevine physiological indicator, the irrigation scheduling was managed in a precise manner, as the irrigation criteria were based on the grapevine water demand rather than on soil moisture measurements (Acevedo-Opazo et al., 2010). Water stressed plants were left to dehydrate until $\Psi_{STEM}$ dropped down to -1.2 MPa to -1.4 MPa. Then the water stress was partially released by supplying water (13 July, 2012). Additionally, to desiccate three healthy and three GFLV-infected grapevines, they were left to dry (Table 3).

- Measurements of Roth hydraulic conductivity

The RHC ($nL\ MPa^{-1}\ s^{-1}\ g^{-1}\ DW$) was measured with the pressure-flux technique. Grapevines with intact roots that were still in the soil were inserted into a pressure chamber filled with water. The plant stem was cut, inserted in the upper hole and the chamber was closed. A pressure of 0.3 MPa was gradually applied inside the chamber, and at the top of the stem that remained, the sap was collected for three periods of 3 min each. At the end, the roots were washed and weighted for fresh and dry matter, with partitioning together with the leaves and shoots.

The shoots and root fresh weights were recorded for each of the individual grapevines. The dry weight of the plant tissue was the weight after drying in an oven for 48 h at 105 °C. The RHC was calculated based on the dry root weight ($nL\ H_2O\ s^{-1}\ g^{-1}\ dw\ MPa^{-1}$). This parameter was measured before the water stress at two time points, during water stress, and after the recovery from the water stress.
Table 3: Schematic representation of the course of the stem water potential, root hydraulic conductivity, water treatments, and sampling points for the gene analysis in the pot experiment in 2012.

<table>
<thead>
<tr>
<th>Day</th>
<th>time points</th>
<th>watering</th>
<th>Measurements of SWP &amp; sampling</th>
<th>RHC</th>
<th>qPCR gene analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>rooting of the shoots</td>
<td>March</td>
<td>April</td>
<td></td>
</tr>
<tr>
<td>pd</td>
<td>1</td>
<td>all plant</td>
<td>6/19/2012</td>
<td>6/19/2012</td>
<td></td>
</tr>
<tr>
<td>d0</td>
<td>2</td>
<td>all plant</td>
<td>6/26/2012</td>
<td>6/26/2012</td>
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<tr>
<td>d3</td>
<td>3</td>
<td>only WW</td>
<td>6/30/2012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d6</td>
<td>4</td>
<td>only WW</td>
<td>7/3/2012</td>
<td></td>
<td>7/3/2012</td>
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<td>5</td>
<td>only WW</td>
<td>7/6/2012</td>
<td></td>
<td>7/6/2012</td>
</tr>
<tr>
<td>d12</td>
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<td>all plant</td>
<td>7/9/2012</td>
<td>7/9/2012</td>
<td>7/9/2012</td>
</tr>
<tr>
<td>d15</td>
<td>7</td>
<td></td>
<td>7/13/2012</td>
<td>7/13/2012</td>
<td></td>
</tr>
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</table>

3.8. Analyses of the presence of viruses by ELISA

Selected plants for analyses of the GFLV titres were tested for the presence of GFLV, *Arabis mosaic virus* (ArMV), *Grapevine fleck virus* (GFkV), *Grapevine virus A* (GVA), and *Grapevine leafroll associated viruses*- 1, -2, -3, and -6 (GLRaV-1, -2, -3, -6), using DAS-ELISA (Bioreba). For the analysis of *Grapevine virus B* (GVB), PTA-ELISA was used (Agritest) according to the producer instructions (Zhang et al., 1998). These were carried out twice, once in January for the phloem scrapings, and once in June for the shoots. The optical densities of all of the samples were measured at 405 nm. The ELISA readout was considered positive when values >2-fold the mean value of the negative controls were reached. For the negative controls, an additional seven grapevines of Refošk that tested negative for all of the tested pathogens, were used. The presence of the nematode vector was shown in the vineyards in Dutovlje, Vrhpolje and Kromberk.

The samples collected in vineyards for the monitoring of SWP and RHC were analysed using ELISA (Bioreba kits) for the presence of *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine leafroll associated virus* (GLRaV)-1, -2, -3, -4-9, *Grapevine virus A* (GVA), and *Grapevine fleck virus* (GFkV). The grapevines selected for the measurements of plant water status and for the gene analyses were also tested for *Grapevine virus B* (GVB), *Tomato black ring virus* (TBRV), *Grapevine chrome mosaic virus* (GCMV) (Agritest test),
Tomato ringspot virus (ToRSV), Raspberry ringspot virus (RpRSV), Strawberry latent ringspot virus (SLRSV) and Tobacco ringspot virus (TRSV), as described by Cigoj (2015).

Buffers for ELISA

Extraction buffer (pH 8.2):

- TRIS (Sigma, Germany) 264 mM
- TRIS-HCl (Sigma, Germany) 236 mM
- NaCl (Merck, Germany) 137 mM
- PVP K25 (Fluka, Germany) 2%
- PEG 6000 (Merck, Germany) 2 mM
- Tween 20 (Sigma, Germany) 0.05%

Phosphate-buffered saline (PBS) washing buffer (pH 7.4):

- NaCl (Merck, Germany) 137 nM
- KH₂PO₄ (Ridel, Germany) 1.5 nM
- Na₂HPO₄ (Merck, Germany) 8 nM
- KCl (Merck, Germany) 3 nM
- Tween 20 0.05%

Coating buffer (pH 9.6):

- Na₂CO₃ (Merck, Germany) 15 nM
- NaHCO₃ (Merck, Germany) 35 nM

Conjugate buffer for DAS ELISA (pH 7.4):

- TRIS (Sigma, Germany) 20 nM
- NaCl (Merck, Germany) 137 nM
- PVP K25 (Fluka, Germany) 2%
• Tween 20 0.05%
• BSA (Sigma, Germany) 0.2%
• MgCl₂ x 6H₂O (Merck, Germany) 1 nM
• KCl (Merck, Germany) 3 nM

Substrate buffer (pH 9.8):
• Diethanolamine 9.7%

Conjugate buffer for DASI-ELISA (pH 7.4):
• NaCl (Merck, Germany) 137 nM
• KCl (Merck, Germany) 3 nM
• PVP K25 (Fluka, Germany) 2%
• BSA 0.02%
• Tween 20 0.05%
• KH₂PO₄ (Ridel, Germany) 1.5 nM
• Na₂HPO₄ (Merck, Germany) 8 nM

Leaves from the apical shoots were ground in Bioreba bags with the extraction buffer (1:10; w/v) using a Homex grinder (Bioreba, Nylon, Switzerland). The DAS-ELISA procedure was used for the majority of the tested viruses, except for GVB, where DASI-ELISA was used.

• DAS-ELISA

The antibodies (200 µL) (Bioreba AG, Switzerland or Agritest, Italy) diluted in coating buffer (1:1000) were added to 96-well plates (Greiner). The plates were covered and incubated for 4 h at 30 °C for the Bioreba antibodies and for 2 h at 37 °C for the Agritest antibodies. After the incubation, the 96-well plates were washed for four times with washing buffer.

The homogenised plant material (200 µL) was added to the 96-well plates, which were incubated overnight at 4 °C. The next day, the 96-well plates were washed four times with washing buffer. After washing, 195 µL antibodies (Bioreba AG, Switzerland Agritest, Italy)
diluted in conjugate buffer (1:1000) were added. After 5 h of incubation at 30 °C for the Bioreba antibodies, and after 2 h at 37 °C for the Agritest antibodies, the 96-well plates were washed four times with washing buffer. After that, 200 µL of 1 mg/mL para-nitrophenyl-phosphate in substrate buffer was added. The 96-well plates were then incubated at room temperature. The optical density was measured at 405 nm (OD\textsubscript{405}) after 30 min, 1 h, 2 h and 18 h of incubation with the substrate, using a plate reader (Tecan Sunrise™, Männedorf, Switzerland). The data were processed using the Magellan™ data analysis software. The ELISA readout was considered positive when values >2-fold of the value of the negative controls were reached.

**DASI-ELISA**

The homogenised plant material (200 µL) was added to 96-well plates (Greiner), which were covered and incubated overnight at 4 °C. After this incubation, the 96-well plates were washed four times with washing buffer. Two hundred microlitres of primary antibodies (Agritest, Italy) diluted in conjugate buffer (1:1000) were added. The 96-well plates were covered and incubated for 2 h at 37 °C. After the incubation, the 96-well plates were washed four times with washing buffer. Then 200 µL of secondary antibodies (Agritest, Italy) diluted in conjugate buffer (1:1000) were added. After 2 h of incubation at 37 °C, the 96-well plates were washed four times with washing buffer. After that, 200 µL of 1 mg/mL para-nitrophenyl-phosphate in substrate buffer was added. The 96-well plates were incubated at room temperature. The OD\textsubscript{405} was measured after 30 min, 1 h, 2 h and 18 h of incubation with the substrate, using a plate reader (Tecan Sunrise™, Männedorf, Switzerland). The data were processed using the Magellan™ data analysis software. The ELISA readout was considered positive when values >2-fold the value of the negative controls were reached. For each sample, the mean OD\textsubscript{405} was calculated. The inhibition was excluded by diluting a pool of extracts in the extraction buffer at 1:10, 1:10\textsuperscript{2}, 1:10\textsuperscript{3}, 1:10\textsuperscript{4}, 1:10\textsuperscript{5} and 1:10\textsuperscript{6}.
3.9. Relative quantification of GFLV by semi-quantitative ELISA

Two-hundred-and-fifty milligrams fresh grapevine material was ground in Bioreba bags with grapevine extraction buffer (1:10; w/v) prepared according to the Agritest (Valenzano, Italy) protocol, using a Homex grinder, and then stored at -80 °C until use. For the DAS-ELISA, anti-GFLV-IgG antibodies (Bioreba AG, Switzerland) were used according to the manufacturer instructions. OD$_{405}$ was measured after 30 min and 1 h of incubation with substrate (1mg/mL paranitrophenyl phosphate) using a plate reader (Tecan Sunrise™, Männedorf, Switzerland). The data were processed using the Magellan™ data analysis software. Inhibition was excluded by diluting a pool of extracts in the extraction buffer in the ratios 1:10, 1:10$^2$, 1:10$^3$, 1:10$^4$, 1:10$^5$ and 1:10$^6$. For the first three of these dilutions, a proportional decline in the logarithm OD$_{405}$ was observed. The virus titre was calculated based on a normalisation curve.

3.10. Relative quantification of the GFLV titre by RT-qPCR and gene expression analyses

The grapevine material was stored at -80 °C until it was used for RNA extraction. For the relative quantification of the GFLV titre, 250 mg of grapevine material was ground in Bioreba bags with RLC extraction buffer (Qiagen, Chatsworth, CA, USA) preheated to 56 °C and containing 10 mg/mL PVP MW 40000 (Sigma) at 1:14 (w/v), using a Homex grinder (Bioreba, Nylon, Switzerland). One millilitre of the extract was incubated for 3 min at 56 °C, and then centrifuged for 30 s at 10,000× g, according to Hren et al. (2009).

For total RNA extraction for gene analyses, the grapevine leaves were ground to a fine powder in liquid nitrogen and weighed. Subsequently, 100 ±20 mg was placed into tubes and RLC extraction buffer was added. These samples were the vortexed vigorously, incubated for 3 min at 56 °C, and centrifuged for 30 s at 10,000× g.

The supernatants obtained were used in the subsequent steps of RNA extraction, using the RNeasy Plant Mini kits (Qiagen, Chatsworth, CA, USA) according to the manufacturer
instructions. Five hundred microliters of lysate (supernatant) was transferred to a QIA-shredder spin column (purple), which was then centrifuged for 2 min at 14,000× g. This step was repeated. After centrifugation, both of the lysates were combined in a new microcentrifuge tube, and 0.5 volume of ethanol (Sigma, Germany) was added and mixed immediately by pipetting.

Then 650 µL of the samples was transferred to an RNeasy spin column (pink), which was centrifuged for 20 s at 10,000× g. The flow-through was discarded, and the steps were repeated until the whole volume of each sample was used. The spin-column membrane was washed once with 700 µL RW1 buffer and twice with 500 µL RPE buffer to remove all of the remaining proteins and impurities. The RNA was eluted twice using 30 µL RNase free water that had been preheated to 65 °C each time, with a 5-min incubation at room temperature (T = 23 °C ±2 °C) in between, and then stored at -80 °C until the analyses were carried out.

For the gene expression analyses, the RNA was additionally quantified using a Nanodrop spectrophotometer (NanoDrop Technologies). For the samples collected in the pot trial, RNA quality and concentration was measured using an Agilent 2100 bioanalyzer, using 2 µl of each sample with Agilent RNA 6000 Nano kits.

**DNase treatment**

DNase treatment was carried out using DNase I, Amplification Grade, kits (Invitrogen, USA).

**The reaction mixture for each sample contained:**

- DNase I, Amplification Grade 0.1 µL
- 10× DNase I reaction buffer 2 µL
- RNase free water 8 µL
- RNA 10 µL

These samples were incubated at room temperature (T = 23 °C ±2 °C) for 15 min. After this incubation, the reaction mixture was inactivated with 2 µL 25 nM EDTA solution, and heated for 10 min at 65 °C. Then 12.5 µL of each sample was denatured for 5 min at 80 °C and placed on ice.

Reverse transcription was carried out with High Capacity cDNA Reverse Transcription kits (Applied Biosystems, USA).
The 2× reverse transcription master mix for each sample contained:

- 10× reverse transcription buffer: 2.5 µL
- 25 nM dNTP mix: 1 µL
- Reverse transcription random primers: 2.5 µL
- RNase free water: 4.25 µL
- RNase inhibitor: 1 µL
- MultiScribe™ reverse transcriptase: 1.25 µL

The master mix (12.5 µL) was added to the DNase-treated samples (12.5 µL) and processed on a GeneAmp® PCR System 9700HT (Applied Biosystem), as follows: 25 °C for 10 min, 37 °C for 120 min, and 4 °C for ∞.

3.11. Quantitative polymerase chain reaction

For analysis of the GFLV titre, 10-fold and 100-fold diluted RNA was used in duplicate for TaqMan® one-step reverse transcription real-time PCR (RT-qPCR) testing (Čepin et al., 2010). As reference genes for normalisation selected were cytochrome oxidase (COX) and 18S rRNA. The validation of their stability of expression was carried out using geNorm (Pfaffl, 2001a; Vandesompele et al., 2002a), which calculates the gene-stability measure M for both reference genes in a given set of samples.

The relative quantification of the GFLV titre was expressed as the ratio (r) between the GFLV concentration in each sample and the GFLV concentration in a defined control sample. Cq values were normalised to the geometric mean of the expression of both of the reference genes (Vandesompele et al., 2002a). The relative expression ratio was calculated based on the efficiencies of amplification (Equation 1) of each amplicon in each sample:

\[
E = 10^{(1/\text{slope})}
\]

where the slope represents ΔCq between the 10-fold and 100-fold dilutions and the differences of the normalised Cq values between each individual sample and control sample (Pfaffl, 2001a).
The $r$ value (Equation (2)) is proportional to the number of viral RNA2 molecules in each analysed sample. Sample amplification efficiencies and the normalisation with reference genes excluded potential intersample variations due to differences in the efficiency of RNA isolation, reverse transcription, and the amplification itself.

\[ r = \text{ratio} = \frac{(E_{GFLV})^{\Delta C_q \text{ GFLV (control-sample)}}}{(E_{\text{reference}})^{\Delta C_q \text{ reference (control-sample)}}} \quad (2) \]

where $E_{GFLV}$ is the efficiency of GFLV amplification in each individual sample; $E_{\text{reference}}$ is the efficiency of reference gene amplification in each individual sample, calculated from the geometric mean of the $COX$ and 18S $C_q$ values; $\Delta C_q \text{ GFLV}$ is the $C_q$ deviation of the GFLV amounts between control sample and an individual sample; $\Delta C_q \text{ reference}$ is the $C_q$ deviation of the amounts of the reference genes (geometric mean of $COX$ and 18S $C_q$ values) between the control sample and an individual experimental sample.

For gene expression analysis, the qPCR method was selected. Each reaction (5 µL) contained 2 µL cDNA and 3 µL mastermix (SYBR® Green or TaqMan), 300 nM of each primer for the SYBR Green chemistry, and 300 nM primers and 150 nM probes for the TaqMan chemistry. For the SYBR® Green chemistry, the Power SYBR® Green PCR Master Mix (Applied Biosystems, USA) was used. For the TaqMan chemistry, the TaqMan Universal PCR Master Mix (Applied Biosystems, USA) was used.

As reference genes for normalisation, $COX$ and ubiquitin-conjugating factor ($UBI\_CF$) were selected. The validation of the stability of their expression was carried out using geNorm (Pfaffl, 2001; Vandesompele et al., 2002), which calculated the gene stability measure for both of the reference genes in a given set of samples.

For the genes $NCED1$, $NCED2$, $RD22$, $WRKY$, $OLP$, $SAMT$, chalcone synthase ($CHS2$), flavanone 3-hydroxylase 1 ($F3H1$), flavanone 3-hydroxylase 2 ($F3H2$), flavonoid 3’ hydroxylase ($F3’H$), flavonoid 3’ 5’ hydroxylase ($F3’5’H$), $LDOX$, $LAR2$ and $UBI\_CF$, the SYBR® Green chemistry was selected, while for $COX$, the TaqMan chemistry was used. The primer pairs and probe characteristics for qPCR are shown in Table 4.
Table 4: Primer pairs used in the analysis of gene expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide sequence 5´-3´</th>
<th>Sequence</th>
<th>Final concentration (nM)</th>
<th>Reference</th>
</tr>
</thead>
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<td>OLP</td>
<td>Forward TCGCCAGTCTAAAACCTACTAGG</td>
<td>300</td>
<td></td>
<td>Nikolić, 2011</td>
</tr>
<tr>
<td></td>
<td>Reverse CGTAAAGAAGGTGTTTTCATGAG</td>
<td>300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMT</td>
<td>Forward TCCCTTACCATAAGTAGGGCTGG</td>
<td>300</td>
<td></td>
<td>Nikolić, 2011</td>
</tr>
<tr>
<td></td>
<td>Reverse TCCGCACCTGCTCTCATC</td>
<td>300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCED1</td>
<td>Forward TCCCTACAGGACCTCCTATG</td>
<td>300</td>
<td></td>
<td>Soar et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Reverse ATATGCGGACCATCCCTCT</td>
<td>300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCED2</td>
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<td></td>
<td>Reverse GGAAGATCCAAAGAGGGAAA</td>
<td>300</td>
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<td></td>
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<td>RD22</td>
<td>Forward CCCATCCTTGCTCTATCTCT</td>
<td>300</td>
<td></td>
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<tr>
<td></td>
<td>Reverse CACCCTCAGGTCTCATTTC</td>
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<td>CHS2</td>
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<td>200</td>
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<tr>
<td></td>
<td>Reverse AGGGTAGCTGCGTAGGTTGG</td>
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<td></td>
</tr>
<tr>
<td>F3H1</td>
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<td></td>
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<tr>
<td></td>
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<tr>
<td>F3H2</td>
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<td>300</td>
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<tr>
<td></td>
<td>Reverse CAATGGTCTAGCGGACTGC</td>
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<td>WRKY</td>
<td>Forward TCCCCATATGAGAAAGGGAAGGG</td>
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<td>Castellarin et al., 2006</td>
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<td></td>
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<td>LAR2</td>
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<td></td>
<td>Reverse TGCAGTTCCTTTTAGTGTTTC</td>
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<td></td>
<td>Reverse AGCCCAATCTTGGGAAGCA</td>
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<tr>
<td>UBI_CF</td>
<td>Forward CTATATGCTCTGCTGACG</td>
<td>300</td>
<td></td>
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<tr>
<td></td>
<td>Reverse AAGCCAGCCAGAGACAACTC</td>
<td>300</td>
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<tr>
<td>GFLV</td>
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<td>900</td>
<td></td>
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<td></td>
<td>Probe 2 FAM-ATGCTCAAACTTG-AGCTTGC</td>
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</tr>
<tr>
<td></td>
<td>Reverse TCATCACTTGTGACAGCTACTTCTT</td>
<td>900</td>
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<td>COX</td>
<td>Forward CGTCCGATTCGAGATATCCA</td>
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<td>Weller et al., 2000</td>
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<td></td>
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<tr>
<td></td>
<td>Probe TGCTTACGGCTGAGATGCGCT</td>
<td>150</td>
<td></td>
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</tr>
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</table>

Three microlitres of Mastermix was pipetted into each well of 384-well plates (384 Well Clear Optical Reaction Plates, Applied Biosystems, USA), and then 2 μL cDNA was added. For each sample, 10-fold and 100-fold diluted cDNA was used in technical duplicates. For each amplicon, the non-template control (NTC) was carried out using water instead of cDNA.

After pipetting, the wells were covered with an adhesive cover (Thermo Scientific) and centrifuged for 1 min at 1000× g. The qPCR was carried out in a LightCycler® 480 instrument.
The qPCR cycles were performed as follows: 50 °C for 2 min, 95 °C for 10 min (polymerase activation), and then 40 cycles at 95 °C for 10 s and 60 °C for 1 min. For SYBR Green chemistry, the dissociation curve (95 °C for 15 s, 60 °C for 15 s, 95 °C for 15 s) was performed to verify the specificity of the products and primer dimers. The initial data analysis was performed with the Roche LightCycler Software, and then the Cq values were exported to Excel files for further analysis.

The relative quantification of the samples with the calibration curve was used. For each amplicon, the calibration curve was constructed. The relative expression ratio was calculated based on the efficiencies of amplification of each amplicon in each sample, where the slope represents ∆Cq between 10-fold and 100-fold dilutions, and the differences of the normalised Cq values between each individual sample and the control sample. The Cq values were normalised to the geometric mean of the expression of the two reference genes (COX, UBI_CF).
3.12. Data analysis

The data were divided in groups according to cultivar, training system, health status, and water treatment. The analysis of the means, standard errors, coefficients of variation (CVs), correlation coefficients, and intervals of confidence of the samples was carried out using Microsoft Excel 2010.

The variability of the GFLV titre was expressed according to the CV (Equation (3)), which allows comparisons in the variability regardless of the magnitude of the analyte concentration (Reed et al., 2002). The CV of the ELISA and RT-qPCR results were calculated as the ratios of the standard deviations (σ) and the mean (µ), multiplied by 100:

\[
\text{CV} (\%) = \frac{\sigma}{\mu} \times 100
\]  
(3)

For the grapevines infected only by GFLV, the dependence between the intensity of symptoms and the GFLV titres was analysed by calculating the correlation coefficient (R²) between the number of different symptoms recorded during the three whole growing seasons and the GFLV titres for five time points in one of those growing seasons (from June to September GFLV titres were measured for shoots, and in January GFLV titres were measured for phloem scrapings). Correlation coefficients were calculated from 23 individual grapevines of ‘Refošk’, ‘Malvazija’ and ‘Volovnik’; and from the means of the grapevines of each of these three cultivars (‘Refošk’, ‘Malvazija’ and ‘Volovnik’).

The statistical analysis was carried out using Welch two sample t-tests and using One-way and/or Two-way Analysis of Variance tests (Statgraphics 5.1. Statpoint Inc., Virginia, USA) to determine the statistically significant differences between the samples according to \( p = 0.05 \), as the limit for statistical significance. When differences were statistically significant, multiple Student–Newman–Keuls (SNK) comparisons were used to separate the significantly different means from each other. The statistically significant differences between the healthy and GFLV-infected plants are indicated as *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \).

The principal component analysis (PCA) method was used to analyse the grapevine water status and the gene expression. The data of the gene expression were transformed with log2. Vector length identified variability and the contribution of the gene to the formulation of the main
component (factor). To find patterns in the data and to classify any combinations of variables that could explain the effects of GFLV infection and gene expression during the season for the field-growing grapevines, the dataset used for the PCA analysis contained the four time points in which the variables of 11 genes were analysed. PCA was also used to obtain a hierarchy of the variables analysed, to find patterns in the data, and to classify any combinations of variables that might explain the effects of GFLV infection and irrigation treatments on gene expression. Therefore, the dataset used for the PCA analysis contained the three time points (d0, d9, d12) and the irrigation treatments (H-ww, H-ws, I-ww, I-ws), which were analysed as variables for 12 genes.
4. RESULTS

4.1. Investigation of a large number of grapevines for the presence of viruses

From 240 tested grapevines with symptoms of GFLV infection and grapevines with no symptoms as samples that were collected in Slovenia (Karst region, Vipava Valley) and in Italy (Prepotto), 61 grapevines were positive for GFLV, 17 for GFkV, one for GVA, and eight for GLRaV-1, -2, -3. In 10 of the grapevines, mixed infections were present, while 153 samples were negative for all of the tested viruses. No infections with ArMV and GLRaV-4-9 were observed (Figure 5).

![Diagram of grapevine virus infections](image)

Figure 5: Diagrams of the grapevine virus infections for the tested viruses: GFLV, GVA, GFkV, GLRaV-1, -2, -3, -4, -9 and ArMV for the grapevines collected in Slovenia (Karst region, Vipava Valley) (A) and the health status for the grapevines tested, as the healthy and infected grapevines (B).

In previous studies, some samples were tested also for the presence of phytoplasma and bacteria. No phytoplasma or bacteria were detected in these analysed samples. On the basis of these results, the vineyards and grapevines were selected for the further target analyses.
4.2. GFLV symptoms expressions on GFLV-infected grapevines

A total of 20 different symptoms were observed on the GFLV-infected grapevines, which were: malformations of leaves (e.g., fan leaves, asymmetrical leaves, sharply toothed leaf margins, crinkled leaves, smaller leaves), yellowing (e.g., spot chlorosis, chlorotic mottling, yellow mosaic), malformations of shoots (e.g., shoot bifurcations, bifurcation on internodes, bifurcation on nodes, short internodes, double nodes, shoots growing together, flattened stems, zig-zag growth, dense leaf growth, bushy like growth, shortened shoots) and yield reduction (Figure 6).

More than 80% of the GFLV-infected grapevines expressed symptoms. The most frequent symptoms recorded on the GFLV infected grapevines were leaf deformation (e.g., asymmetrical leaves), double nodes, short internodes, malformed shoots with abnormal branching, and yellow-mosaic-associated symptoms.

Figure 6: Photographs illustrating the symptoms of GFLV on grapevines, showing yellowing (A, B, G), malformations of leaves (C), shoots (D, H, J) and clusters (E, I), as recorded in the vineyards (Jež Krelbelj, 2012).
4.3. Symptoms development and dynamics of GFLV infections in an established vineyard in Vrhpolje and in a replanted vineyard in Dutovlje.

Figure 7: (A) Symptoms of yellow mosaic on ‘Laški rizling’ in the vineyard in Vrhpolje. (B) ‘Refošk’ grapevines planted in the vineyard in Dutovlje (Jež Krebelj, 2011).

In established vineyard in Vrhpolje

In over 10 years of monitoring yellowing symptoms (in the framework of the L4-0165 Research Programme), for 15 grapevines of ‘Laški rizling’ (Figure 7), it was found that the yellowing spreads in a concentrical circle (see below). Grapevines in the centre of the yellowing origin are mainly all GFLV infected, grapevines planted on the edge of the origin are not GFLV infected. We were interested in the rate of the GFLV infection through the vineyard, and the appearance of yellowing. In 2011, the symptoms of yellowing were monitored throughout the whole vineyard. Grapevines selected on the edge of the yellowing area were sampled, where the last grapevine in a row had symptoms and the next grapevine was without yellowing. Grapevines LR3/28 and LR4/28, which were the closest to the centre of the yellowing origin, expressed symptoms later than was observed in the nearest grapevine, with yellowing of the grapevines beginning in 2011 and 2013, respectively. The same pattern of development of yellowing symptoms was observed also for grapevines LR7/28 and LR10/21. Over these years, we observed that some grapevines irregularly expressed symptoms of yellowing.
Three GFLV-uninfected grapevines in 2011 (LR5/19, LR6/18 and LR7/21) that were tested by ELISA in both years (Figure 8) remained uninfected also in 2013, although the nematode (*X. index*) was shown to be present in the vineyard.

In replanted vineyard in Dutovlje

In 2010, grapevines of ‘Refošk’ were planted in the vineyard in Dutovlje, where GFLV infection was observed in previous studies (L4-0165). Therefore, we investigated symptoms appearance. In addition, ELISA was preform to detected GFLV infection in the planted grapevines. In both years, approximately half of the grapevines tested positive for GFLV, and half negative. Symptoms of yellowing were observed for grapevine 2/46 in 2013, although it was already GFLV infected in 2011. GFLV-infected grapevine 2/43 irregularly expressed yellowing over the years. Grapevines with GFLV infection were more concentrated at the right side of the row (Figure 11), which might indicate the incidence of GFLV infection by *X. index* or the infected plant material was used (the plant material was not tested for the presence of GFLV).

Figure 8: ELISA results for GFLV-positive (+) and GFLV-negative (-) grapevines of ‘Laški rizling’, tested in 2011 and 2013, according to symbols indicated. Red boxes, grapevines tested in both years; empty boxes, grapevines not tested.

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Figure 9: ELISA results for GFLV positive (+) and negative (-) grapevines of ‘Refošk’, tested in 2011 and 2013, according to symbols indicated. Red boxes, grapevines tested in both years; empty boxes, grapevines not tested.
Figure 10: Schematic representation of the data of symptoms appearance and ELISA results for ‘Laški rizling’ from 2004 to 2013. Each ‘slice’ of the circle represents a year. Yellow, leaf yellowing; orange, yellow mosaic (spot chlorosis); green, grapevines without yellowing; black, grapevines eliminated from the vineyard or dead; G, GFLV positive; --, GFLV negative.
Figure 11: Schematic representation of the data of symptoms appearance and ELISA results for ‘Refošk’ from 2011 to 2013. Each ‘slice’ of the circle represents a year. Yellow, leaf yellowing; orange, yellow mosaic (spot chlorosis); green, grapevines without yellowing; G, GFLV positive; --, GFLV negative.
4.4. Fluctuations in the virus titre during the season for the different parts of the grapevines, and dynamics of GFLV infection in established vineyards and in newly replanted vineyards

4.4.1. Variability of GFLV titre within the grapevines

In grapevines 1-6, the absence of infection for all of the tested pathogens except GFLV was shown. The same results were determined for 24 out of 53 additional grapevines of cultivars ‘Refošk’, ‘Volovnik’, ‘Malvazija’ and ‘Župlanka’, while the remaining 30 analysed grapevines showed mixed infections of GFLV and one or more other viruses, including: GFkV, GLRav-1, -2, -3 and/or GVA.

To evaluate the variability of the GFLV titre within the grapevines, multiple samples were collected from each grapevine at the same time and analysed by qPCR and ELISA. The variability of the GFLV titre was analysed in phloem using qPCR. The lowest variability of the GFLV titre was shown between samples of phloem taken from the same part of the cane at the same time point (CV = 10%), which confirmed the accuracy of the method (Figure 12A). On average, an approximately 3-fold greater GFLV titre was found for the basal part of the canes than for the apical part (p = 0.012), and this was reflected in the greater variability between the samples of phloem from the basal, medium and apical parts of the canes taken at the same time point (CV = 16%) (Figure 12B). The variability of the GFLV titre between the samples of phloem from different grapevines at the same time point in the season was only slightly higher (CV = 18%) than between samples from the same grapevine at the same time point in the season (Figure 10C), but much

lower than the variability between samples from different time points in the season (CV ≤43%), which indicated a greater impact on the GFLV titre in phloem of season than sampling position.

The variability of the GFLV titre in phloem, in young and mature leaves, and in tendrils was analysed using ELISA, to compare the samples from the basal, medium and apical parts of the canes collected at the same time. In phloem, the data obtained by ELISA were more variable than those obtained by qPCR. This was due to the semi-quantitative nature of ELISA, which allows rough estimations and comparisons of the virus titres. The ELISA results showed a CV of 66% for the GFLV titres between the samples of phloem, in comparison to a CV of 16% measured using qPCR. The variability of the GFLV titre between the samples of young and mature leaves (CV ELISA = 42% and 46%, respectively) and between samples of tendrils (CV ELISA = 25%) was lower than between the samples of phloem.
Figure 12: Variability of the GFLV titre in phloem at the same time point. (A) Variability between the samples of phloem from the same part of the canes for five grapevines (grapevines 1-5) in three different parts of the cane: basal, part a (red); middle, part b (orange); apical, part c (yellow). (B) Variability between the samples of phloem from different parts of the canes for five grapevines (grapevines 1-5). (C) Variability between the samples of phloem taken from five different grapevines. Symbols represent means; bars represent minimum and maximum.

4.4.2. Fluctuations in the GFLV titre through the season

Fluctuations in GFLV were monitored using ELISA during the vegetative period (monthly, from May to September) for phloem, young and mature leaves, tendrils, flowers/berries and roots, and outside the vegetative period (in January) for phloem and roots. In phloem, the fluctuations in GFLV were additionally monitored by qPCR. By qPCR, the highest virus titres in phloem were
observed at the beginning of the vegetative period (in May) and outside the vegetative period (in January). During the summer, the virus titres were statistically significantly lower (Figure 13A). A similar trend in the lowest GFLV titres in phloem during the summer period was observed also using ELISA, although the differences were not statistically significant, probably due to the lower accuracy of ELISA compared to qPCR (Figure 13B).

Figure 13: GFLV titre in phloem through the season measured by qPCR (A) and ELISA (B). Mean r values and OD values are shown for qPCR and ELISA, respectively. Error bars represent standard errors. Different letters indicate statistically significant differences obtained by t-test. (p <0.05).

By ELISA, statistically significant fluctuations in the virus titres through the season were observed in mature leaves, tendrils and flower/ berry clusters. In mature leaves and tendrils, high virus titres were observed in June and July, and these were statistically significantly lower in August and September (Figure 14B, D). In flower/ berries, the virus titre decreased gradually during the vegetative period, and was statistically significantly higher in May and June than in July and August, and was lowest in September (Figure 14C). On the other hand, no statistically significant changes in the virus titres were observed through the season in young leaves and roots (Figure 14A, E).
Figure 14: GFLV titres in young (A) and mature (B) leaves, flowers/berries (C), tendrils (D) and roots (E) through the season, as measured by ELISA. Mean OD values are shown. Error bars represent standard errors. Different letters indicate statistically significant differences obtained by t-test (p < 0.05).
4.4.3. Distribution of GFLV within the grapevines

In young leaves, the virus titre was high throughout the vegetative period (Figure 14A), while it decreased through the vegetative period in flowers/berries (Figure 14C), tendrils (Figure 14D) and mature leaves (Figure 14B). It was also low throughout the vegetative period in phloem (Figure 14B) and roots (Figure 14E), where 12% and 15% of the samples, respectively, even tested negative using ELISA. There were differences in the virus titres between different organs/tissues at certain times through the season. From June to August, the virus titre was statistically significantly lower in phloem than in young leaves, mature leaves, flower/berry clusters and tendrils, and in September, it was lower than in young leaves and tendrils. In June, the virus titre in roots was statistically significantly lower than in young leaves, mature leaves, flower/berry clusters and tendrils, and in August and September, it was lower than in young leaves and tendrils. In August and September, the virus titre in young leaves was statistically significantly higher than in mature leaves, flower/berry clusters, phloem and roots, and in tendrils, it was statistically significantly higher than in phloem and roots (Table 5, Figure 14). In January, there were no statistically significant differences between the virus titre in roots and phloem.
Table 5: Statistical significance for the differences between the GFLV titres in young and mature leaves, flower/ berry clusters, tendrils, phloem and roots, in June, July, August and September obtained by t-test. *** p ≤0.001; ** p ≤0.01; * p ≤0.05; empty field, no statistical significance seen.

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4.5. Distribution of GFLV in berries

An uneven distribution of GFLV was found in berries by measuring the GFLV titre in skins, flesh and seeds of not yet coloured (green) and already coloured (red) berries. In both, green and red berries, the virus titre in seeds was statistically significantly higher than in flesh and skins. In green berry skins, the virus titre were even under the limit of detection using the ELISA method. On the other hand, the proportions between virus titres in seeds and flesh remained constant through both developmental phases (Figure 15).
Figure 15: GFLV titre in green and red berries, measured by ELISA. Average OD values are shown. Error bars represent standard errors. Different letters indicate statistically significant differences obtained by t-test (p <0.05).

4.5.1. Comparison of GFLV titres between different grapevine cultivars

To compare GFLV titres between the different grapevine cultivars, shoots of five cultivars were sampled monthly from June to September and then tested by ELISA: ‘Župlanka’, ‘Malvazija’, ‘Laški rizling’, ‘Volovnik’ and ‘Refošk’. In ‘Župlanka’ and ‘Malvazija’, the virus titre was high through the whole vegetative period, while in ‘Refošk’, ‘Volovnik’ and ‘Laški rizling’ the virus titre decreased statistically significantly in the second half of the vegetative period (Figure 16).

Figure 16: GFLV titre in shoots, measured by ELISA. Average OD values are shown. Error bars represent standard errors. Different letters indicate statistically significant differences obtained by t-test (p <0.05).
4.5.2. Correlation between GFLV titres and GFLV symptoms

The numbers of disease symptoms were counted for each of the 23 grapevines (of cvs. ‘Refošk’, ‘Volovnik’, ‘Malvazija’) infected with GFLV (and that had tested negative for all of the other viruses). Between ‘Malvazija’ and ‘Volovnik’, there were no statistically significant differences in the numbers of disease symptoms (p = 0.116), while there were statistically significantly lower numbers of disease symptoms for ‘Refošk’ as compared to ‘Volovnik’ (p <0.001) and ‘Malvazija’ (p = 0.001) (data not shown).

Correlation coefficients were calculated between the numbers of different symptoms recorded through three full growing seasons, and between the GFLV titres in shoots (from June to September) and in phloem scrapings (in January) for one of the growing seasons. The correlation coefficients calculated from the individual grapevines ranged from \( R^2 = 0.01 \) in September, to \( R^2 = 0.44 \) in January (Figure 17). The correlation coefficients calculated from the means of all of the grapevines within each cultivar (i.e., ‘Refošk’, ‘Volovnik’, ‘Malvazija’) were higher, and ranged from \( R^2 = 0.02 \) in September, to \( R^2 = 0.95 \) in July.

![Scatterplot between the number of symptoms](image.png)

**Figure 17:** Scatterplot between the numbers of symptoms recorded through the whole growing season and the GFLV titres in phloem scrapings in January in individual grapevines (●), and means of ‘Refošk’, ‘Volovnik’ and ‘Malvazija’ (□). R\(^2\), correlation determination
4.5.3. Influence of mixed infections on GFLV titres in cultivar ‘Refošk’

In ‘Refošk’, the GFLV titres in shoots were compared between grapevines infected with GFLV and grapevines infected with combinations of GFLV and one (GLRaV-1 or GLRaV-2) or two (GLRaV-1 and GLRaV-3, or GLRaV-1 and GVA, or GFkV and GLRaV-3) other viruses, through the vegetative period (monthly, from June to September), using ELISA. In all of these combinations of infections, the GFLV titre decreased gradually during the vegetative period (Figure 18). Different combinations of mixed infections influenced the GFLV titre differently, although only the co-infection with GLRaV-2 in August influenced the GFLV titre statistically significantly when compared to the GFLV titre in grapevines infected only with GFLV.

Figure 18: GFLV titres in shoots of cultivar ‘Refošk’ infected with GFLV or a combination of GFLV and one or two other viruses, as measured by ELISA. Average OD values are shown. Error bars represent standard errors.
4.6. Physiological responses and gene expression of *Vitis vinifera* of 'Schioppettino' and ‘Refošk’ monitored in the vineyard subjected to grapevine water status changes and GFLV infection

4.6.1. Agronomical characteristics of the grapevines

The numbers of buds (Figure 19) and blind buds (Figure 20) measured at the beginning of the summers of 2011 and 2012 in 45 grapevines of ‘Schioppettino’ and in 16 grapevines of ‘Refošk’ were not different between healthy and GFLV-infected grapevines. Also, the numbers of clusters (Figure 21) and the numbers of shoots (Figure 22) in ‘Schioppettino’ showed no differences between healthy and GFLV-infected grapevines for both of the training systems. However, for ‘Refošk’, the mean numbers of clusters and shoots were reduced in the GFLV-infected grapevines, as compared to the healthy grapevines, for both of these years, although the differences did not reach statistical significance.

During the winter of 2011/2012, using the single Guyot training system for both of these cultivars (i.e., ‘Refošk’, ‘Schioppettino’), pruning weight (Figure 24) was lower in GFLV-infected grapevines compared to healthy grapevines. In ‘Schioppettino’, the difference was statistically significant for grapevines trained using the single Guyot system, while in the double Guyot training system, no differences was seen. For both of these cultivars using both of these training systems, the mean lengths of internodes (Figure 23) of GFLV-infected grapevines was shorter than in the healthy grapevines, although again, this difference did not reach statistical significance.

For the single Guyot training system for both of these cultivars (i.e., ‘Refošk’, ‘Schioppettino’), the mean Ravaz index in 2011 was lower in the infected grapevines compared to the healthy grapevines. However, the mean Ravaz index of grapevines trained using the double Guyot training system was a little higher (Figure 25).
Figure 19: Numbers of buds in healthy and GFLV-infected grapevines of ‘Schioppetino’ using the single Guyot (SG) and double Guyot (DG) systems, and for ‘Refošk’, in seasons 2011 and 2012. Differences between means established using t-tests. Error bars represent standard errors.

Figure 20: Numbers of blind buds in healthy and GFLV-infected grapevines of ‘Schioppetino’ using the single Guyot (SG) and double Guyot (DG) systems, and for ‘Refošk’, in seasons 2011 and 2012. Differences between means established using t-tests. Error bars represent standard errors.
Figure 21: Numbers of clusters in healthy and GFLV-infected grapevines of ‘Schioppetino’ using the single Guyot (SG) and double Guyot (DG) systems, and for ‘Refošk’, in seasons 2011 and 2012. Differences between means established using t-tests. Error bars represent standard errors.

Figure 22: Numbers of shoots in healthy and GFLV-infected grapevines of ‘Schioppetino’ using the single Guyot (SG) and double Guyot (DG) systems, and for ‘Refošk’, in seasons 2011 and 2012. Differences between means established using t-tests. Error bars represent standard errors.
Figure 23: Internode lengths of healthy and GFLV-infected grapevines of ‘Schioppetino’ using the single Guyot (SG) and double Guyot (DG) systems, and for ‘Refošk’ in 2011. Differences between means established using t-tests. Error bars represent standard errors.

Figure 24: Pruning weights of healthy and GFLV infected grapevines of ‘Schioppetino’ using the single Guyot (SG) and double Guyot (DG) systems, and for ‘Refošk’ in 2011. Significant differences between grapevines were obtained using t-tests; p \leq 0.029. Error bars represent standard errors.
Figure 25: Mean Ravaz index (yield weight/pruning weight) of the healthy and GFLV-infected grapevines that were monitored in ‘Schioppettino’ using the single Guyot (SG) and double Guyot (DG) systems, and for ‘Refošk’ for the 2011 vintage. Error bars represent CV ($\sigma/\mu$).
4.6.2. Stem water potential in the field

The SWP was measured in the same two cultivars (i.e., ‘Refošk’, ‘Schioppettino’) in their locations, to define the water status conditions through the season at the different development stage of the grapevines: BBCH 53, inflorescences clearly visible; BBCH 77, berries beginning to touch; BBCH 81, beginning of ripening (berries begin to develop variety-specific colour); and BBCH 89, berries ripe for harvest.

The differences between healthy and GFLV-infected grapevines for SWP were measured from the beginning of July 2011 to the beginning of September 2011, and in 2012 from the beginning of July to the beginning of September.

For the 2011 season, the SWP of GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot system was statistically significant lower compared to healthy grapevines for the first three time points (Figure 26B). In healthy grapevines, the SWP ranged from -0.46 MPa to -0.91 MPa, while in the GFLV-infected grapevines, the SWP ranged from -0.51 MPa to -1.00 MPa. In the grapevines of ‘Schioppettino’ trained using the double Guyot system, the GFLV-infected grapevines had lower SWP compared to the healthy ones, although no statistically significant differences for the SWP were obtained between healthy and infected grapevines (Figure 26C). The SWP ranged from -0.57 MPa to -0.86 MPa in healthy grapevines, and from -0.63 to -0.91 MPa in GFLV-infected grapevines. Over the course of the measurements, the SWP of ‘Refošk’ (REF K9T) ranged from -0.41 MPa to -0.96 MPa in healthy grapevines and from -0.42 to -1.01 MPa in infected grapevines. The GFLV-infected grapevines had lower SWP compared to the healthy grapevines, although the differences were not statistically significant (Figure 28).

For the 2012 season, the SWP of GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot system was statistically significantly lower compared to the healthy grapevines for the first two time points. Here, the SWP ranged from -0.55 MPa to -1.04 MPa, while for the infected grapevines it ranged from -0.59 MPa to -1.10 MPa over the course of the measurements (Figure 27B). The SWP of healthy grapevines of ‘Schioppettino’ trained using the double Guyot system (Figure 27 C) ranged from -0.65 MPa to -1.13 MPa, while for the infected grapevines SWP ranged
from -0.72 MPa to -1.19 MPa, over the course of the measurements. Although the difference was not statistically significant, the GFLV-infected grapevines had lower SWP compared to the healthy ones. The SWP of the healthy grapevines of ‘Refošk’ (REF K9T) trained using the single Guyot system (Figure 29) ranged from -0.43 MPa to -1.00 MPa, while for the infected grapevines, the SWP ranged from -0.48 MPa to -1.03 MPa, over the course of the measurements. In the other vineyard, the SWP of healthy plants of ‘Refošk’ (REF 26) trained using the single Guyot system (Figure 30) ranged from -0.63 MPa to -0.97 MPa, and the SWP of infected grapevines ranged from -0.76 MPa to -1.03 MPa. Although the differences were not statistically significantly, the GFLV-infected grapevines had lower SWP compared to the healthy grapevines.
Figure 26: Trends in the SWP of grapevines grown in the field, for ‘Schioppettino’ in 2011. (A) Mean SWP of all of the grapevines of ‘Schioppetino’. (B, C) Mean SWP of the grapevines trained using the single Guyot system (B) and the double Guyot system (C). Differences between means established using t-tests. *** (p ≤ 0.001), ** (p ≤ 0.01), *(p ≤ 0.05). Error bars represent standard errors.
Figure 27: Trends in the SWP of grapevines grown in the field, for ‘Schioppettino’ in 2012. (A) Mean SWP of all of the grapevines of ‘Schioppettino’. (B, C) mean SWP of the grapevines trained using the single Guyot system (B) and the double Guyot system (C). Differences between means established using t-tests. *** (p ≤0.001), ** (p ≤0.01), *(p ≤0.05). Error bars represent standard errors.
Figure 28: Trends in the SWP of grapevines grown in the field for ‘Refošk’ (REF K9T) in Komen in 2011. Error bars represent standard errors. Differences between means established using t-tests; n.s. (p >0.05).

Figures 29: Trends of SWP of grapevines grown in the field for ‘Refošk’ (REF K9T) (A) and ‘Refošk’ (REF 26) (B) in Komen in 2012. Error bars represent standard errors. Differences between means established using t-tests.
4.7. Gene expression

To evaluate the grapevine response to drought (an abiotic stress) and virus infection (a biotic stress), expression of the *NCED1*, *NCED2* and *RD22* genes were studied in selected plants, using RT-qPCR. The expression of the *WRKY*, *SAMT*, *OLP* and *SuSy* genes were also determined. Finally, the expression of the *CHS2*, *F3H1*, *F3H2* and *LAR2* genes were determined to monitor the activity of the flavonoid pathway.

*NCED1* was highly expressed in leaves of ‘Schioppettino’ trained using the double Guyot training system for both healthy and infected plants, while the data for *NCED2* for ‘Schioppettino’ trained using the double Guyot system were not obtained.

![NCED1 gene expression](image)

Figure 30: *NCED1* gene expression in grapevine leaves of healthy and GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot training system (SCH SG) and the double Guyot training system (SCH DG), and for ‘Refošk’ (REF K9T, REF 26), for the four time points during the vegetative season in 2012. Differences between means established using *t*-tests.***, *p* ≤0.001; **, *p* ≤0.01; *, *p* ≤0.05. Error bars represent standard errors.
In the ‘Schioppettino’ trained using the single Guyot system and in ‘Refošk’, NCED1 gene expression was lower compared to ‘Schioppettino’ trained using the double Guyot system (Figure 30). NCED1 expression was also higher for the fourth time point (beginning of September) regardless of GFLV infection.

In general, GFLV infection did not affect NCED1 and NCED2 expression, except that NCED1 in ‘Refošk’ (REF K9T) and NCED2 in ‘Refošk’ (REF K9T, REF26) were slightly down regulated in GFLV-infected grapevines, compared to healthy controls, although these changes were not statistically significant (Figure 31).

![Figure 31: NCED2 gene expression in grapevine leaves of healthy and GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot training system (SCH SG) and the double Guyot training system (SCH DG), and of ‘Refošk’ (REF K9T, REF 26), for the four time points during the vegetative season in 2012. Differences between means established using t-tests. ***: p ≤0.001; **: p ≤0.01; *: p ≤0.05. Error bars represent standard errors.](image)

In the leaves of ‘Schioppettino’ trained using the single Guyot system, and of ‘Refošk’ (REF26), regardless of GFLV infection, NCED2 expression was higher at the fourth time point, compared to the earlier sampling times.
GFLV infection did not affect the expression of the *RD22* gene, except in ‘Schioppettino’ trained using the double Guyot system, where *RD22* was up-regulated in infected grapevines at the first two time points and down-regulated for the last two time points in GFLV-infected leaves, compared to healthy controls (Figure 32). The down-regulation at the fourth time point reached statistical significance.
In ‘Schioppettino’, regardless of the grapevine infection, the expression of the \textit{WRKY} gene was higher at the first sampling point, following by a reduction in \textit{WRKY} expression through the next two sampling points, and with higher expression again for the fourth sampling point.

In ‘Refošk’, \textit{WRKY} was down-regulated in GFLV-infected grapevines compared to healthy controls in both vineyards and at all four time points. For the first sampling point, the down-regulation of \textit{WRKY} in ‘Refošk’ REF K9T was statistically significant (Figure 33).

The expression patterns of the \textit{SAMT} and \textit{OLP} genes were very similar (Figures 34, 35). \textit{SAMT} (i.e., S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase (O-methyltransferase)) (Ament et al., 2010; Tieman et al., 2010) is the key enzyme in the synthesis of methylsalicylate (MeSA) from salicylic acid, and the expression of \textit{SAMT} and \textit{OLP} were in general higher for the first and/or last time points than for the other time points.
Figure 34: SAMT gene expression in grapevine leaves of healthy and GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot training system (SCH SG) and the double Guyot training system (SCH DG), and of ‘Refošk’ (REF K9T, REF 26) for the four time points during the vegetative season in 2012. Differences between means established using t-tests . *** p ≤ 0.001; ** p ≤ 0.01; * p ≤ 0.05. Error bars represent standard errors.

In ‘Refošk’, SAMT and OLP were generally down-regulated in GFLV-infected leaves compared to healthy controls. The exception was the first time point, and for OLP in REF K9T also the second time point, when OLP was up-regulated. These changes in SAMT and OLP expression were not statistically significant.
Figure 35: OLP gene expression in grapevine leaves of healthy and GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot training system (SCH SG) and the double Guyot training system (SCH DG), and of ‘Refošk’ (REF K9T, REF 26) for the four time points during the vegetative season in 2012. Differences between means established using t-tests. ***, p ≤0.001; **, p ≤0.01; *, p ≤0.05. Error bars represent standard errors.

There was no large influence of GFLV infection on the expression of the SuSy gene. However, in ‘Refošk’ (REF 26), where the data for the first time point are missing, the expression of SuSy in both cultivars and using both training systems was higher at the beginning of the growing season than for the later time points. In ‘Refošk’, a non-significant down-regulation was seen for the third and fourth time points in both of the vineyards.
Figure 36: SuSy gene expression in grapevine leaves of healthy and GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot training system (SCH SG) and the double Guyot training system (SCH DG), and of ‘Refošk’ (REF K9T, REF 26) for the four time points during the vegetative season in 2012. Differences between means established using t-tests. ***, p ≤ 0.001; **, p ≤ 0.01; *, p ≤ 0.05. Error bars represent standard errors.

At the start, the SuSy gene was up-regulated in grapevines of ‘Schioppettino’ trained using the single Guyot system, regardless of the healthy status. Expression of SuSy in ‘Schioppettino’ trained using the double Guyot and in ‘Refošk’ (REF K9T) was also up-regulated in grapevines at the start of the vegetative period (Figure 36). The opposite expression of SuSy was seen for the infected grapevines of ’Refošk’ (REF 26). At the start of the vegetative period, SuSy was down-regulated in these grapevines, following by increased of expression, regardless of infection status. The SuSy gene was slightly less expressed in infected grapevines.
Figure 37: CHS2 gene expression in grapevine leaves of healthy and GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot training system (SCH SG) and the double Guyot training system (SCH DG), and of ‘Refošk’ (REF K9T, REF 26) for the four time points during the vegetative season in 2012. Differences between means established using t-tests. ***, p ≤ 0.001; **, p ≤ 0.01; *, p ≤ 0.05. Error bars represent standard errors.

As a member of the CHS family, the CHS2 gene is involved in the first step in the flavonoid pathway, and it was statistically significantly down-regulated in GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot system, for the first sampling point, and in ‘Refošk’ (REF26) for the second time point, where the data for the first sampling point are missing (Figure 37). In ‘Schioppettino’ trained using the double Guyot training system and in ‘Refošk’ (REF K9T), there were no differences in the expression pattern of CHS2 between the healthy controls and the virus infection.

In general, the expression patterns of the flavanone 3-hydroxylase (F3H) genes F3H1 and F3H2 were similar for ‘Schioppettino’, regardless of the training system, and in ‘Refošk’ (Figures 38, 39). In ‘Schioppettino’ trained using the single Guyot system, the expression of F3H1 was statistically significantly up-regulated in GFLV-infected grapevines for the fourth time point compared to healthy grapevines, while in ‘Refošk’ (REF K9T, REF 26), F3H1 was statistically significantly down-regulated in the GFLV-infected grapevines compared to the healthy grapevines. In ‘Schioppettino’ and in ‘Refošk’, the expression pattern of F3H2 was similar to that obtained for
*F3H1*, although the differences between healthy grapevines and GFLV-infected grapevines were not statistically significant.

Figure 38: *F3H1* gene expression in grapevine leaves of healthy and GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot training system (SCH SG) and the double Guyot training system (SCH DG), and of ‘Refošk’ (REF K9T, REF 26) for the four time points during the vegetative season in 2012. Differences between means established using *t*-tests. ***, *p* ≤0.001; **, *p* ≤0.01; *, *p* ≤0.05. Error bars represent standard errors.

Figure 39: *F3H2* gene expression in grapevine leaves of healthy and GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot training system (SCH SG) and the double Guyot training system (SCH DG), and of
‘Refošk’ (REF K9T, REF 26) for the four time points during the vegetative season in 2012. Differences between means established using *t*-tests. ***, *p* ≤ 0.001; **, *p* ≤ 0.01; *, *p* ≤ 0.05. Error bars represent standard errors.

![Figure 39: LAR2 gene expression in grapevine leaves of healthy and GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot training system (SCH SG) and the double Guyot training system (SCH DG), and of ‘Refošk’ (REF K9T, REF 26) for the four time points during the vegetative season in 2012. Differences between means established using *t*-tests. ***, *p* ≤ 0.001; **, *p* ≤ 0.01; *, *p* ≤ 0.05. Error bars represent standard errors.](image)

Regardless of GFLV infection, the LAR2 gene had similar trends for its expression in ‘Schioppettino’ trained using the single Guyot and in ‘Refošk’ (REF K9T, REF 26) for both of the vineyards (Figure 40). While in ‘Schioppettino’ trained using the double Guyot system LAR2 was higher expressed in GFLV-infected grapevines in all four of the time points, in healthy plants the expression of LAR2 was irregular. There was no statistically significant difference in the expression of LAR2 between the healthy and GFLV-infected grapevines.

Furthermore, we wanted to determine whether based on the relative expression of 11 target genes, these grapevines can be separate by their GFLV status (i.e., healthy and infected), also according to sampling point (July-September), and finally we wanted to determine the genes with the most impact on the grouping of these samples. The data were thus analysed using principal component analysis (PCA).
For ‘Schioppettino’ trained using the single Guyot system, 32.56% of the variation in the data can be explained by principal component 1, and 24.47% of the variability by principal component 2. Therefore, both of these components accounted for 57.03% of the total variability in the data. Principal components 1 and 2 both contributed to the separation between healthy and GFLV-infected grapevines (Figure 41A). Interestingly, the NCED1, NCED2, SAMT, WRKY and SuSy genes all grouped tightly together, and these contributed the majority of the variability explained by principal component 1. Instead, the CHS2, F3H1 and F3H2 genes are all involved in the anthocyanin biosynthetic pathway, and these grouped tightly together and contributed the majority of the variability explained by principal component 2 (Figure 41B). The gene that are involved in the same metabolic pathway were grouping together. In the fourth time point samples were grouped tightly together due to the expression of the NCED1, NCED2, SAMT, OLP and WRKY.
Figure 41: (A) Principal component analysis of the healthy (green) and GFLV-infected (red) grapevines from the vineyard in Prepotto, for ‘Schioppettino’ trained using the double Guyot training system, and sampled during the vegetative season in 2012 for the four sampling points. (B) Principal component analysis for 11 genes of the grapevines from the vineyard in Prepotto, for ‘Schioppettino’ trained using the double Guyot training system.

For ‘Schioppettino’ trained using the double Guyot training system, 33.12% of the variation was expressed by component 1, and 26.09% of the variation by component 2. Therefore, both of these components accounted for 59.21% of the total variability in the data. Similar to ‘Schioppettino’ trained using the single Guyot system, in case of double Guyot the genes NCED1, SAMT, OLP, WRKY and SuSy genes contributed to the majority of the variability explained by principal component 1, and the CHS2, F3H1 and F3H2 genes contributed to the majority of the variability explained by principal component 2 (Figure 42B). The expression of the genes was noisy due to the variability of the phonological synchronicity of the vines, which was consequence of the higher variability of the vine population. This resulted in less tight grouping of vine samples in different time points.
Figure 42: (A) Principal component analysis of the healthy and GFLV-infected grapevines from the vineyard in Komen, for ‘Refošk’ (REF 26) trained using the Sylvoz training system and sampled during the vegetative season in 2012 for the three sampling points. (B) Principal component analysis for 11 genes of the grapevines from the vineyard in Komen, for ‘Refošk’ (REF 26).

For ‘Refošk’ (REF 26) from the vineyard in Komen, 57.33% of the variation was explained by principal component 1, and 16.96% of the variation by principal component 2. Therefore, both of these components accounted for 74.29% of the total variability in the data. The healthy and GFLV-infected grapevines were separated by principal component 2 (Figure 43A). The majority of the variation explained by principal component 2 derived from the NCED1, NCED2, SAMT, OLP, WRKY and SuSy genes, while the CHS2, F3H1 and F3H2 genes contributed to the variation explained by principal component 1 (Figure 43B). Same pattern as was observed for ‘Schioppettino’ trained using the single Guyot system, also grouped together the gene that are involved in the same metabolic pathway. Also, in the fourth time point samples were grouped tightly together due to the expression of the NCED1, NCED2, SAMT, OLP and WRKY.
Figure 43: (A) Principal component analysis for the healthy and GFLV-infected grapevines from the vineyard in Komen, for 'Refošk' (REF K9T) trained using the single Guyot training system and sampled during the vegetative season in 2012 for the four sampling points. (B) Principal component analysis for 11 genes of the grapevines from the vineyard in Komen, for 'Refošk' (REF K9T).

For 'Refošk' (REF K9T) from the vineyard in Komen, 40.35% of the variation was explained by principal component 1, and 16.46% of the variation by principal component 2. Therefore, both of these components accounted for 56.81% of the total variability in the data. The CHS2, F3H1 and F3H2 genes were not so clearly separated from the other genes as they were in the other vineyards (Figure 44B). Vines was less tight grouping together in different time points as it was observed in case of REF26 and the gene expression was noisy. This, can be related to biological variability between vines.
4.8. Physiological impact of GFLV virus infection on grapevine water status and drought under controlled conditions

4.8.1. Growth parameters

The effects of drought were quantified through the effects on growth (i.e., fresh and dry weights of roots, fresh and dry weights of shoots, leaf area) in the own-rooted plants of ‘Schioppettino’ in pots (Figure 44). The GFLV-infected grapevines here had higher fresh weights of roots compared to the healthy grapevines. Before the water stress was applied (first and second time points) the difference was statistically significant. At day six, the water-stressed grapevines had statistically significantly higher fresh root weight.

Figure 44: Potted grapevines of ‘Schioppettino’ used for the measurements of SWP and RHC after planting in pots in April of 2012.
Figure 45: Root fresh and dry weights and water content of the ‘Schioppettino’ grapevines in pots. Differences between means established using t-tests. ***, p ≤ 0.001; **, p ≤ 0.01; *, p ≤ 0.05.
The GFLV-infected grapevines had higher root dry weights compared to healthy grapevines. At the start (the first time point, when no water stress was applied), the difference was statistically significant. At day 12, the water-stressed grapevines had statistically significantly higher fresh root weights compared to the well water grapevines (Figure 45). The GFLV-infected grapevines also had, in general, slightly higher root water content compared to healthy grapevines. Lower water content was observed for the roots of the water stressed plants compared to the well-watered plants after 12 days of water stress (Figure 45).
Figure 46: Shoot fresh and dry weights and water content of the ‘Schioppettino’ grapevines in pots. Differences between means established using t-test ***, p ≤0.001; **, p ≤0.01; *, p ≤0.05.
In general, higher fresh shoot weights were observed in the healthy grapevines compared to the GFLV-infected grapevines. After 6 days of water reduction, the water-stressed grapevines had statistically significantly higher fresh shoot weights compared to the well-watered grapevines.

Similar to the fresh weigh of the shoots, the dry weights of the shoots of the healthy grapevines were also higher than those of the shoots of the GFLV-infected grapevines. After 6 days of water stress, the water-stressed grapevines had higher dry weights compared to the well-watered grapevines. No significant influence of either GFLV infection or water stress on the water content in the shoots was found.

Figure 47: The numbers of leaves on the shoots of healthy and GFLV-infected grapevines of cultivar ‘Schioppettino’. Differences between means established using t-tests (Statistics; StatSoft, USA).***, *p ≤0.001; **, p ≤0.01; *, p ≤0.05. Error bars represent standard errors. (●) GFLV-infected, well-watered grapevines; (●) healthy, well-watered grapevines; (●) GFLV-infected and treated with 12 days of watered stress, and re-watered on day 12; (●) healthy grapevines treated with 12 days of water stress.

In general, the GFLV infected grapevines had lower numbers of leaves compared to the healthy grapevines (Figure 47). The mean lengths of the main veins on the leaves were statistically significantly lower in the GFLV-infected leaves on day 0 (Figure 48).
4.8.2. Water status and root hydraulic conductivity

The study of the impact of GFLV infection and drought on the SWP and RHC in these own-rooted grapevines of ‘Schioppettino’ showed that there was an impact of GFLV infection on SWP to a certain level of water deficit. When the grapevines were exposed to stronger water stress, the effects of GFLV infection were negligible compared with the effects of the water depletion (Figure 49B). Statistically significant differences between the well-watered and water-stressed plants were seen below a certain level of water deficit (Figure 49A).
Figure 49: Evolution of SWP before water stress, during water stress, and after recovery, as affected by the water stress (A) and the GFLV infection (B). The time period of the water deficit is indicated by the shading. Differences between means established using t-tests. ***, p ≤ 0.001; **, p ≤ 0.01; *, p ≤ 0.05. Error bars represent standard errors.
The RHC was reduced with the water-stress treatment compared to the control grapevines (Figure 50A). The RHC of the infected grapevines were also lower than for the healthy grapevines (Figure 50B). Interactions between these factors were seen. There was a higher impact of the virus infection than the water stress. After the recovery, there were no differences in RHC between infected and healthy plants.
Figure 50: Evolution of RHC before the water stress, during the water stress, and after the recovery, as affected by water stress (A) and GFLV infection (B). The time course of the water deficit is indicated by the shading. Differences between means established using t-tests. ***, p ≤ 0.001; **, p ≤ 0.01; *, p ≤ 0.05. Error bars represent standard errors.
4.8.3. Gene expression in young leaves of healthy and GFLV-infected grapevines in the pot trial

The interactions between the GFLV-infected and water-stressed grapevines and the expression of genes in the young leaves in healthy and GFLV-infected grapevines were analysed using two-ways ANOVA.

The water deficit did not change the expression of the CHS2, LAR2, SAMT, OLP and SuSy genes. The F3H1, F3H2, NCED1, NCED2 and WRKY genes were down-regulated after 9 days of water stress (when SWP reached -0.7 MPa) and were up-regulated after 12 days of water stress (when SWP reached -1.1 MPa). The up-regulation of F3H2, LDOX, RD22 and WRKY after 12 days of water stress was statistically significant (Figures 51, 52).

The GFLV infection statistically significantly influenced the expression of NCED1, NCED2, CHS2 and F3H1. For the other genes, there was a slight up-regulation of OLP and SuSy for GFLV infection at day zero (with no water stress), when RD22 was down-regulated. After 9 days of water stress, CHS2 and NCED1 were down-regulated in GFLV-infected grapevines compared to healthy grapevines, under both conditions (i.e., well watered, water stress) (Figure 51). After 12 days of water stress, LDOX and OLP were slightly up-regulated, and RD22 was down-regulated, in GFLV-infected grapevines compared to healthy grapevines, under both conditions (i.e., well watered, water stress).
Figure 51: Gene expression for grapevines of ‘Schioppettino’ planted in pots, for *NCED1*, *NCED2* and *RD22* (A) and *SAMT*, *OLP* and *SuSy* (B) in the leaves, for plant water status (regardless of healthy and GFLV infection; white background) and for sanitary status (regardless of well-watered and water-stressed conditions; shaded background). Differences between means established using *t*-tests . *** p ≤0.001; ** p ≤0.01; * p ≤0.05. Error bars represent standard errors. (●) well watered regardless of the GFLV infection and health status of the grapevines; (●) water stressed regardless of the GFLV infection and health status of the grapevines; (Δ) healthy grapevines regardless of the water status (WW, WS); (▲) GFLV-infected grapevines regardless of the water status (WW, WS).
Figure 52: Gene expression for grapevines of ‘Schioppettino’ planted in pots, for CHS2, F3H1, F3H2 (A) and LAR2, LDOX, WRKY in the leaves, for plant water status (regardless of healthy and GFLV infection; white background) and for sanitary status (regardless of well-watered and water-stressed conditions, shaded background). Differences between means established using *t*-tests. ***; p ≤0.001; **; p ≤0.01; *; p ≤0.05. Error bars represent standard errors. (◌) well watered regardless of the GFLV infection and health status of the grapevines; (●) water stressed regardless of the GFLV infection and health status of the grapevines; (Δ) healthy grapevines regardless of the water status (WW, WS); (▲) GFLV-infected grapevines regardless of the water status (WW, WS).
These data were confirmed also by PCA. At day zero (with no waters stress), 65.36% of the variation in the data was explained by component 1, and only 15.52% of the variation in the data was explained by component 2. All of the investigated genes contributed to the variability explained by principal component 1 (Figure 53B). Principal component 1 was negatively correlated with the majority of the target genes, and positively correlated with RD22.

There was clear separation between healthy and GFLV-infected grapevines according to component 1 (Figure 53A), which means that all of the investigated genes contributed to the separation. The additional variability of each gene was calculated. Both RD22 and WRKY were higher than 0.7 and were therefore considered as statistically significant (Supplementary Table 2).

After nine days of water stress, 37.12% of the variation in the data was explained by principal component 1, and 31.49% of the variation by principal component 2 (Figure 54). Therefore, both components accounted for 68.61% of the total variability in the data. There was clear separation

Figure 53: Principal component analysis (PCA) of the 12 expressed genes. Scaled expression data relative to the healthy and GFLV-infected grapevines according to day zero (A) and the GFLV infection (second principal component) (B). (●) GFLV-infected, well-watered grapevines; (○) healthy, well-watered grapevines; (●) GFLV-infected grapevines treated with 12 days of watered stress (●) healthy grapevines treated with 12 days of watered stress.
between healthy and GFLV-infected grapevines according to component 2 (Figure 54A). The CHS2, F3H2, NCED1 and NCED2 genes contributed the majority of the variability that was explained by principal component 2 (Figure 54B).

Component 2 was positively correlated with the CHS2, F3H2, NCED1 and NCED2 genes. The variability of these genes was higher or very close to 0.7, and therefore this was considered as statistically significant (Supplementary Table 2). No clear separation was observed between water-stressed and well-watered grapevines, as the majority of the genes that were differentially regulated by water stress (i.e., F3H1, F3H2, NCED1, NCED2, WRKY) were differently regulated by GFLV infection at the same time.

Figure 54: Principal component analysis (PCA) of the 10 expressed genes. Scaled expression data relative to the healthy and GFLV-infected grapevines are according to day 9 (first principal component) (A), and according to GFLV infection (second principal component) (B). (●) GFLV-infected, well-watered grapevines; (○) healthy, well-watered grapevines; (●) GFLV-infected grapevines treated with 12 days of water stress; (●) healthy grapevines treated with 12 days of water stress.
After 12 days of water stress, 58.16% of the variation in the data was explained by principal component 1, and 16.25% by principal component 2 (Figure 55). Therefore, both components accounted for 74.41% of the total variability in the data. There was clear separation between healthy and GFLV-infected grapevines according to principal component 2, and separation between water-stressed and well-watered grapevines according to component 1.

Component 2 was negatively correlated with LDOX and OLP expression and positively correlated with RD22 expression. Component 1 was positively correlated with the expression of all of the other investigated genes (i.e., F3H1, F3H2, WRKY, SuSy, NCED1, NCED2, CHS2, SAMT, LAR2) (Figure 55B). The variability of these genes was statistically significant (Supplementary Table 2).

Figure 55: Principal component analysis (PCA) of the 12 expressed genes. Scaled expression data relative to the healthy and GFLV-infected grapevines according to day 12 (first principal component) (A) and to GFLV infection (second principal component) (B). (●) GFLV-infected, well-watered grapevines; (●) healthy, well-watered grapevines; (●) GFLV-infected grapevines treated with 12 days of water stress; (●) healthy grapevines treated with 12 days of water stress.
5. DISCUSSION

5.1. Symptoms development and the dynamics of GFLV infection in the established vineyard in Vrhpolje and in the replanted vineyard in Dutovlje.

The study of the dynamic of GFLV infection in replanted vineyard in the presence of GFLV vector *X. index* in the soil, showed that the infection could be detected in some vines already one year after planting. The infection could either be the consequence of planting the GFLV infected material (we did not have possibility to test the planting material) or the consequence of infection of the vines by GFLV infected nematodes that were present in the vineyard (the presence of the nematodes was shown and before planting the new vineyard, a GFLV infected vines was planted in the same soil). In next two years none of the newly infected vines was detected. Similar observation was observed in the already established vineyard, only one new infected grapevine was found over the seven-year period, although *X. index* was present in the soil and the concentric patch of GFLV infection was clearly seen. Although, only one newly infected vine was found in seven years, new appearances of the symptoms were observed in several vines. In some vines symptoms were observed in the third, fourth and fifth year of the observation for the first time. In vineyard in Dutovlje the symptoms were seen very rarely already in the first year of the infection, which might indicate that newly planted vines are more susceptible for the infection than older and that mature-plant resistance (MPR) was present to some extent. Some of the symptoms were expressed as severe malformations and deformations of leaves and shoots and/or leaf yellowing, and some of the grapevines had less expressed GFLV symptoms or were symptomless. The factors affecting symptom expression are unknown, but they might include the virus strain, host genotype, and environmental conditions, and perhaps the effects of other viruses that simultaneously infect the grapevine (Martelli and Savino, 1988). In vineyards, GFLV infection and symptoms expression might be correlated with the presence of *X. index*, as was reported by Villate et al. (2008), where nematode patches correlated significantly with the position of the grapevines with yellowing symptoms.
5.2. Distribution of GFLV within grapevines and fluctuations in the GFLV titre through the season

The distribution of GFLV within the grapevines and the fluctuations in the GFLV titre through the season were determined. The highest GFLV titre was observed at the beginning of the vegetative period (in May and June) in young leaves and tendrils. In young leaves, the GFLV titre remained high throughout the whole vegetative period, and therefore it is recommended that young leaves are used as the testing material for the diagnosis of GFLV when sampling during the vegetative period (Figure 14A, D).

Rowhani et al. (1992) came to similar conclusions, as they detected the maximum amounts of GFLV in young leaves and shoot tips of Vitis rupestris ‘St. George’ early in the growing season (May-June), although in their study the virus titre in the young leaves and shoot tips dropped in July. In the present study, a decrease in the GFLV titre was observed in mature leaves and tendrils in August and September (Figure 14B, D). Despite the decrease in the GFLV titre, we detected GFLV in all of the tested mature leaves using ELISA, for ‘Refošk’ and ‘Volovnik’. Similar to our observations, Walter and Etienne (1987) successfully determined GFLV using ELISA in grapevine leaves that were collected from a single grapevine throughout the growing season (from May to October), regardless of whether the leaves were sampled at the bottom or the top of the shoot. However, in the study of Bouyahia et al. (2003), samples taken during the vegetative period from marginal areas of the leaf blade of mature leaves from GFLV-infected grapevines gave 80% confidence for a positive result by ELISA, and samples taken from the basal part of the leaf (near the petiole) gave only 10%.

The most problematic tissue/organ for GFLV diagnosis during the vegetative period turned out to be phloem and roots, as 12% of phloem samples and 15% of root samples from GFLV-infected grapevines taken from June to September tested negative using ELISA, while in the qPCR analysis all of these samples were positive. During the summer period (June to September), virus titres in
phloem determined by qPCR were lower than in the beginning of the vegetative period in May, and outside the vegetative period in January (Figure 13A). A similar trend of the lowest GFLV titres in phloem during the summer period was observed also using ELISA, although the differences were not statistically significant, probably due to the lower sensitivity and accuracy of ELISA compared to qPCR (Figure 13B). Similarly, Rowhani et al. (1992) observed relatively constant virus titres in phloem during the vegetative period using ELISA, although in contrast to the present data, they found it to be lower outside the growing season (November to March). In the present study, all phloem and root samples from GFLV-infected grapevines taken out of the vegetative period tested positive using ELISA. Therefore phloem is a useful testing material for diagnosis of GFLV when sampling out of the vegetative period (roots are less convenient to sample). As the virus titres in phloem are lower than in young leaves, the use of qPCR diagnostic test instead of ELISA improves the sensitivity of the analyses.

On the other hand, the monthly analyses of the different plant parts (e.g., opening buds, tips of shoots, unfolded leaves, leaf petioles, completely expanded leaves, green phloem, cortical scrapings) from three GFLV-infected grapevines of cultivar ‘Cabernet sauvignon’ in Chile were positive in 94% and 77% of samples tested using ELISA and RT-PCR, respectively (Fiore et al., 2009). These data can be explained by the high sequence variability of the GFLV genome among the different GFLV isolates. Nucleotide sequence differences were found to range by up to 17%, and amino-acid sequence differences by up to 9% (Mekuria et al., 2009; Oliver et al., 2010; Pompe-Novak et al., 2007; Vigne et al., 2004). Due to this high GFLV nucleotide sequence variability, one-step RT-qPCR assay (i.e., targeting the 2A\textsuperscript{HP} gene) that was validated to reliably detect and quantify different GFLV genotypes from a wide range of geographic regions (Čepin et al., 2010) was used in the present study.

The fluctuation in the GFLV titre in different organs/ tissues through the season might be the consequences of more rapid virus multiplication in metabolically more active young tissues undergoing intensive cell proliferation. These fluctuations in the GFLV titre in different organs/ tissues through the season might also be due to the grapevine defence responses, which might be induced by high levels of viral RNA and/or viral proteins (Jovel et al., 2007). This might activate the silencing processes in certain stages in developing tissues, which might lead to a reduction in
the virus accumulation in these tissues (Siddiqui et al., 2008). Activation of virus-specific RNA silencing has been observed for several plant viruses (Ratcliff, 1997). On the other hand, several plant viruses encode gene-silencing suppressor proteins to overcome the plant defence (Gouveia et al., 2012; Roth et al., 2004; Zhou et al., 2006), although to date, there is no evidence for the presence of a silencing suppressor component in the GFLV genome. Also, young leaves are the sink and mature leaves are the source of the phloem sap, with which the viruses might also be transported. The direction of phloem sap transport from mature leaves to the underground parts of the grapevine through the phloem system would explain the higher virus titres in phloem of the basal parts, in comparison to the apical parts of the canes. This mechanism would also make sense for virus survival, because the leaves drop off in the end of each growing season and the virus stays within the grapevine during winter (in phloem of dormant canes and roots). This explanation is also in line with results of virus titre fluctuations in phloem through the season, where the highest virus titre was found out of the growing season and at the beginning and the end of the growing season, as phloem represents a pool of virus in winter.

Similarly, translocation in the distribution of GLRaV-3 was found through the growing season by RT-qPCR. GLRaV-3 spread quickly from the shoots to new growing canes and leaves at the beginning of the growing season. During late summer and autumn, the virus concentration deceased, but GLRaV-3 infection was still detected in the majority of the plants (Tsai et al., 2012).

The results of the distribution of GFLV within the grapevines and the fluctuations in the GFLV titre through the season were supported by the data on the variability of the GFLV titre in the subsamples. Using qPCR, the lowest variability of the GFLV titre was between samples of phloem taken from the same part of the grapevine canes, which confirms the high accuracy of the method (Figure 12). Higher variability was shown between samples of phloem from the basal, medium and apical parts of the grapevine canes, and an uneven distribution of GFLV in the canes, with the highest GFLV titres in the basal part of the canes, and the lowest GFLV titres in the apical part of the canes. Similarly, also in the study of Bouyahia et al. (2003), more samples from basal internodes of GFLV-infected plants tested GFLV positive than from the apical internodes. The variability of the GFLV titre between phloem from different grapevines at the same time point in the season was only slightly higher than between the same grapevine at the same time point in the season; however,
this was much lower than the variability between grapevines at different time points in the season, which indicates a higher impact of the season than the sampling location on the GFLV titre in phloem.

The data of the GFLV titre in phloem obtained by ELISA were more variable than those obtained by qPCR, due to semi-quantitative nature of ELISA, which allows rough estimations and comparisons of virus titres. From all of the tested organs/ tissues, the variability between samples measured by ELISA was the highest in phloem. Therefore, for the precise quantification and statistical differentiation, these data were complemented by those obtained by qPCR. The variability between the samples from other organs/ tissues measured by ELISA was lower, allowing the calculation of statistically significant differences.

The GFLV titres in shoots were compared between cultivars ‘Župlanka’, ‘Malvazija’, ‘Laški Rizling’, ‘Volovnik’ and ‘Refošk’, monthly during the vegetative period (Figure 16). The amount of GFLV and the dynamics of changes in the GFLV titres were different in these different cultivars. Also the numbers of disease symptoms recorded through the whole growing season were different in these different cultivars. Positive correlation was found between the numbers of symptoms and the GFLV titres. Correlation coefficients calculated from the means of the cultivars were higher than the correlation coefficients calculated from the individual grapevines, probably because the cultivar influences both the expression of symptoms and the virus titre (Figure 17). Positive correlation between symptoms and GFLV titre was also shown by Bouyahia et al.(2003), as 73.6% of their collected symptomatic leaves and only 42.5% of their collected asymptomatic leaves tested positive for GFLV. In contrast, Frantz and Walker (1995) indicated a negative correlation between viral titre and expression of symptoms, although they also implied that the expression of symptoms might also be influenced by the grapevine cultivar, the duration of infection, the number of strains of virus that infected the grapevine, and the presence of the nematode in the vineyard. The effects on GFLV concentration were also related to the location of the infected plant within the vineyard (Pacifico et al., 2011). Differences in symptomatology of GFLVinfected grapevines might also be caused by different physiological responses of the grapevines (Liebenberg et al., 2009) and by differences in the virus strains that infect different grapevines, with variable symptom-eliciting sequences or pathogenicity determinants.
As well as multiple infections in a single grapevine by divergent GFLV isolates (Pompe-Novak et al., 2007; Vigne et al., 2004), mixed infections with other nepoviruses and viruses from different genera are frequent (Laimer et al., 2009). The data for the GFLV titres in mixed infected grapevines indicate that different combinations of mixed infections influence the GFLV titre differently. The titres of five grapevine viruses (i.e., GFLV, GLRaV-1, -3, GVA, GFkV) were measured also in fully expanded basal leaves of ‘Nebbiolo’ in mixed infections with two viruses (GLRaV-1/ GVA, GLRaV-3/ GVA or GFLV/ GFkV). Using RT-qPCR, effects of mixed infections on the multiplication rates of the viruses cannot be excluded (Pacifico et al., 2011).

5.3. Influence of GFLV infection on stem water potential and gene expression of ‘Schioppettino’ and ‘Refošk’ in the vineyard

To investigate the responses elicited by GFLV infection, some important agronomical parameters were evaluated in selected grapevines: grapevine water status, and gene expression of two locally important cultivars, ‘Schioppettino’ and ‘Refošk’.

In the present study, reduced pruning weight and shorter internodes were recorded in the GFLV-infected grapevines compared to the healthy grapevines (Figures 23, 24). In previous studies on the same grapevines, GFLV infection affected the yield. Decreases in berry weight and yield were obtained for both ‘Refošk’ and ‘Schioppettino’ and using both training systems (single and double Guyot). The reductions in the yield were due to smaller berries, and of course to the related lower cluster weights. Also, statistically significant lower cluster weights have been observed in GFLV-infected grapevines (Cigoj, 2015). A reduction in growth was reported for ‘Cabernet Franc’ infected with GLRaV-3 (Endeshaw et al., 2014), for ‘Banyalbufar malmsey’ infected with GFLV, GLRaVs and GFkV (Sampol et al., 2003), and for the autochthonous grapevine varieties of Mallorca cultivars ‘Callet’, ‘Manto Negro’ and ‘Moll’ infected with GFLV and GLRaVs (Cretazzo et al., 2010). Reduction in growth might be caused by a reduction in the grapevine metabolism in the GFLV-infected grapevines.
For both cultivars across both seasons, SWP decreased through the season due to the lowering of the amount of available water in the soil. For both cultivars, both training systems, and both seasons, the SWP of GFLV-infected grapevines was lower than the SWP of healthy plants. The differences in SWP between the healthy and GFLV-infected grapevines were greater in 2011 than 2012, probably because the summer of 2012 was much drier than that of 2011. The differences in SWP between healthy and GFLV-infected grapevines were greater for ‘Schioppettino’ than for ‘Refošk’. This might indicate the genotype-dependent responses to water deficit in grapevines. Some genotypes of V. vinifera have been shown to better control the stomata than others in response to water deficit, and are considered as isohydric. Others have been shown to have lower control over stomatal aperture under water stress, and are considered as anisohydric (Lovisolo et al., 2010).

GFLV also induced changes in the expression of two key genes involved in ABA biosynthesis (NCED1, NCED2), an ABA-responsive gene (RD22), a transcription factor (WRKY), two genes involved stress responses (OLP, SAMT) and four genes involved in the regulation of anthocyanins synthesis (CHS2, F3H1, F3H2, LAR), with these responses shown to be cultivar depended.

The lower expression of NCED in leaves earlier in the season rather than later in the season was probably due to the drying of the soil through the season, as was also reported by Speirs et al., (2013). The induction of NCED1 and NCED2 late in the season in leaves might indicate reduced flow through the vessels during moderate to severe water stress, regardless of the GFLV infection. In response to drought stress, in tomato plants, overexpression of LeNCED1 resulted in increased amounts of ABA (Reguera et al., 2012). The RD22 gene, which responds to dehydration stress in an ABA-mediated manner in other plants (Yamaguchi-Shinozaki and Shinozaki 1993), was statistically significantly down-regulated at the end of the season in GFLV-infected grapevines of ‘Schioppettino’ trained using the double Guyot training system, although this trend was not the case for the other grapevine training system and the other grapevine cultivar.

Transient expression of WRKY54 during the vegetative period might indicate the mechanism of regulating transcription factors, when a stress becomes weak the negative regulator might be required to turn off a tolerant response generated by positive regulators, as has been observed for transcription factors GmWRKY21 and GmWRKY54 involved in drought tolerance (Zhou et al.,
2008). GmWRKY54 might be responsible for stress tolerance, through the regulation of ABA drought-responsive elements in an ABA independent pathway.

The expression patterns of the *SAMT* and *OLP* genes were very similar. In general, they were more highly expressed at the beginning and end of the season than in the middle of the season. Transient expression of *SAMT* might be explained according to the multiple mobile signals for ISR that were observed by Liu et al. (2010). Expression of *SAMT* might depend on various factors, including the plant–pathogen relationship, the developmental stage of the grapevine, and the environmental conditions.

The *OLP* gene codes for thaumatin and osmotin-like protein from group PR-5, and its involvement in grapevine disease responses has already been shown in grapevines infected with BNf and Fdf, where higher expression of *OLP* was found in infected grapevines compared to healthy controls (Albertazzi et al., 2009; Hren et al., 2009; Nikolić, 2011). Expression of *OLP* was induced also in grapevines infected with fungal pathogens (Camps et al., 2010; Spagnolo et al., 2012). Therefore, the expression of *OLP* might be induced as part of a general response of the plant to biotic stress.

Choi et al, (2013) observed coupled expression of sucrose synthase and invertase, which suggested physiological readjustment of leaves from a source of fixed carbon in healthy plants to a sink organ when challenged by *Xylella fastidiosa* or water deficit. Modified expression of *SuSy* was observed in GFLV-infected grapevines at various times through the season. An impact of virus infection on the expression of *SuSy* was also observed in grapevines infected with GLRaVs and GFLV (Nikolić, 2011). Furthermore, *SuSy* was over expressed in grapevines infected with BNf in parallel with symptom induction (Hren et al., 2009a, b; Albertazzi et al., 2009), while grapevines with no symptoms or with mild symptom expression showed no differences in the expression of *SuSy* (Nikolić, 2011).

The early gene in flavonoid biosynthesis, *CHS2*, showed in general similar expression patterns in healthy and GFLV-infected grapevines, although the pattern was shifted in time in the GFLV-infected grapevines compared to the healthy controls. This resulted in statistically significant down-regulation of *CHS2* at the beginning of the season in GFLV-infected grapevines of both cultivars trained using the single Guyot training system. It should be take into account that the
picture of the relative abundance of *CHS* gene transcripts in leaves of GFLV-infected grapevines is not complete, as there is a small gene family of chalcone synthases (CHS1, CHS2, CHS3). *CHS3* expression was shown to be significantly higher than its expression during colour development in GLRAV-3–infected leaves (Gutha et al., 2010).

Very similar expression was seen for the two flavanone-3-hydroxylase isogenes *F3H1* and *F3H2* in healthy and GFLV-infected grapevines. Both of these genes were more highly expressed during the first half of the season than the second half.

The *LAR2* gene is involved in the activity of the proanthocyanidin/catechin pathway, and it was in general slightly down-regulated in GFLV-infected grapevines compared to healthy ones. A total decline in the proanthocyanidins levels has been shown during leaf maturity (Bogs et al., 2005), and *LAR2* was present in different tissues, including leaves. In leaves, the synthesis of the proanthocyanidins was enhanced by GLRaV-3 infection (Gutha et al., 2010), while *LAR2* decreased in berries that were turning red (Castellarin et al., 2007). This indicates the complexity of the regulation of flavonoid biosynthesis under the combination of biotic stresses and environmental stresses that can occur at different times through the vegetative season. Overall, the data indicate specific interactions between the grapevine and GFLV that are synchronised with the environmental (e.g., water supply, water status, temperature) and physiological (e.g., age, training system, development phase) conditions of the grapevine.

Overall, the symptoms represent the sum of the molecular, cellular and physiological changes induced by a virus. Water deficits and GFLV infection alter the expression of genes responsible for some compounds and metabolite transporters in grapevines. Although some of these changes are transient, they have an impact on growth and development of the grapevine. The particularities of the site studied, specifically the RHC, helped to demonstrate the water dynamics of the grapevine. Here, we have shown that the presence of GFLV alone induced a more negative SWP than did deficit irrigation, which suggests that water stress imposed by the pathogen can be locally more severe than that imposed by low water availability in the soil, as was observed by Choi et al. (2013) for treatment effects of GFLV infection and water deficit.
5.4. Influence of GFLV infection and water stress on stem water potential, root hydraulic conductivity, and gene expression in self-rooted plants of ‘Schioppettino’ in pots

Field measurements of $\Psi_{STEM}$ during the vegetative period in 2011 indicated differences in the plant water status between healthy and GFLV-infected grapevines. Therefore, in 2012, a pot experiment was set up to more deeply investigate the relative impact of GFLV infection and water shortage on grapevine physical responses and gene expression.

SWP is an indicator of hydraulic conductivity in the trunk and shoot sap pathway (Choné et al., 2001). A reduction in SWP can be obtained synergistically through the combined impact of low soil water content and cavitation/occlusion of the vessels, which cannot be refilled during the night period (Tyree and Zimmermann, 2002). This reduction also imparts a change to the range of interconnected parameters in a similarly synergic manner.

The responses of grapevines to drought are often associated with an accumulation of ABA in the petiole xylem and leaves (Loveys, 1984; Rodrigues et al., 2008), and consequently with stomatal closure, although increased xylem pH (Rodrigues et al., 2008) and decreased plant hydraulic conductance (Salleo and Gullo, 1989; Vandeleur et al., 2009) might also be involved. In recent studies, it was indicated that hydraulic signals can trigger ABA production in leaves during severe drought stress (Schachtman and Goodger, 2008). Additionally, it was shown that a decrease in the leaf water potential might enhance stomatal sensitivity to ABA (Correia et al., 1995; Rodrigues et al., 2008).

We observed that GFLV infection causes a significant reduction in the SWP and the RHC in well-watered grapevines and through all stages of water deficit. The decrease in RHC in GFLV-infected grapevines compared to healthy grapevines, might be correlated with the concept that the xylem vessels can become disrupted by the breakage of water columns, which causes embolism formation and drastically reduces the hydraulic conductance, as observed in previous studies (Schultz and Matthews, 1988; Lovisolo and Schubert, 1998). Consequently, the SWP of the grapevine might be
decreased by a reduction in hydraulic conductivity. The induction of root and shoot growth in water-stressed grapevines might result from aquaporin stimulation caused by ABA, with the induction of new secondary roots (Kang and Zhang, 2004).

The interrelationships for the plant hydraulics from the roots towards the leaves might be affected by ABA. This might also indicate that the chemical signals are important players in plant adaption to environmental stresses. Moreover, stomatal regulation and photosynthetic assimilation of grapevine is under ABA and hydraulic control (Lovisolo et al., 2010).

In recent studies, SWP and RHC have been shown to have significant importance in the grapevine adaption to severe drought conditions (Schachtman, 2008), while chemical signals (e.g., hormones, transcription factors, metabolites) were shown to be more important under milder water stress.

In the present study, statistically significant influences of water deficit on gene expression were observed only when SWP was under -0.7 MPa, while the influence of GFLV on gene expression was observed regardless of the water status. The expression of NCED1 and NCED2 was not affected by the water deficit. On the other hand, NCED1 and NCED2 in leaves were statistically significantly down-regulated by GFLV infection. Expression studies of the key genes in the ABA biosynthetic pathway have indicated that the regulation of NCED1 gene expression in leaves (but not in roots) is associated with the amount of ABA in the xylem sap. This observation was supported also by an examination of gene expression in leaves and roots from a shadehouse experiment (Soar et al., 2006). Although the genes involved in ABA biosynthesis (NCED1, NCED2) were not regulated by drought, expression of ABA-responsive gene (RD22) and genes involved in the ABA responsive signalling network (WRKY) were significantly affected by severe water deficit.

Nikolić et. al. (2011) reported that grapevine viruses (GFLV, GLRaV, GfKv) induce expression of genes that are involved in the processes of extensive reprograming of the plant transcriptome (SAMT, OLP) in a highly dynamic and temporal manner. Resistance to biotic and abiotic stresses is associated with homeostasis of hormones, such as salicylic acid. Antagonistic cross-talk between ABA (associated with abiotic stress) and salicylic acid (associated with biotic stress) has been observed upon virus, bacteria and fungal infection. In the present study, there were no differences
in the water-stressed and well-watered grapevines for the expression of the \textit{SAMT} and \textit{OLP} genes, while GFLV infection slightly down-regulated \textit{SAMT} and up-regulated \textit{OLP} under conditions of severe water stress. Reduced expression of genes involved in PR-mediated disease responses and increased cytoplasmic protein response was observed under triple stress (drought, heat, virus infection) (Prasch and Sonnewald, 2013).

The early genes of flavonoid biosynthesis include \textit{CHS2}, \textit{F3H1} and \textit{F3H2}, and their expression is essential for further production of flavonols, anthocyanins and proanthocyanidins; these were statistically significantly up-regulated by GFLV infection at day zero (with no water stress), and down-regulated at day 9 (with moderate water stress). Later (with severe water stress at day 12), the expression of these genes was significantly impacted upon by drought. Similar expression pattern of \textit{CHS2} were observed by Castellarin et al. (2007) in grapevines exposed to water stress. Expression of \textit{CHS2} is implicated in the production of proanthocyanidins in unpigmented tissues of grape cultivars, and in leaves and unripe berries. A significant impact of GFLV infection on the expression of \textit{CHS2} might indicate antioxidative responses in an infected leaf. Similar patterns of expression of the \textit{F3H1} and \textit{F3H2} genes were also observed in GLRaV-3–infected leaves (Gutha et al., 2010).

Neither GFLV infection nor water stress affected the expression of \textit{LAR}, which converts leucocyanidin to catechin, which is further converted to epicatechin by an epimerase (Stafford, 1990). A similar observation was reported for grapevine berries in water stressed grapevines (Castellarin et al., 2007), which indicates that the side pathway leading to catechin synthesis that is catalysed by \textit{LAR2} does not have any important role in plant defence responses. On the contrary, it has been shown that the production and accumulation of two isomers of catechins and epicatechins were induced by BNf and FDF infection (Hren et al., 2009 a,b; Nikolić, 2011). On the other hand, \textit{LDOX} is essential for proanthocyanidin synthesis and has an important role in the plant response to water stress. \textit{LDOX} was significantly down-regulated by severe water stress (day 12). We observed also slight, and statistically not significant, up-regulation of \textit{LDOX} by GFLV infection, which indicates greater synthesis of cyanidin, the substrate for anthocyanidin reductase and UFGT. This is consistent with the observation of proanthocyanidin synthesis in \textit{Arabidopsis},
where anthocyanidin reductase uses cyanidin as a substrate, rather than leucocyanidin (Abrahams et al., 2003).

This study has provided some important outlines for a better understanding of the effects of GFLV infection on the grapevine responses to drought conditions. Abiotic and biotic stresses might act synergistically, where the benefits outweigh the costs for both partners. However, studies performed with grapevines grown in pot trials do not always reflect what happens when plants grow in their natural environment. Indeed, parallel studies carried out under control and field conditions provide a wider view of the interactions between viruses and water stress.
In selected vineyards of Slovenian Karst and Vipava Valley and in Prepotto in Italy, there are healthy and GFLV-infected grapevines. As well as the occurrence of GFLV in these tested grapevines, mixed infection with other viruses (GFkV, GVA, GLRaVs) were found. Although X. index (the vector of GFLV) was present in the selected vineyards, we were not able to detect any newly infected grapevines over three years of observations. GFLV was detected also in grapevines that did not express any symptoms through the monitoring period.

Different GFLV titres were shown across five different grapevine cultivars that expressed differently severe disease symptoms, and between grapevines of the same cultivar (‘Refošk’) infected with different virus combinations. The presence of GFLV in all of the analysed tissues, including tendrils, the uneven distribution of GFLV inside the grapevines, and the seasonal fluctuations of GFLV titres in different grapevine tissues are shown here. Young leaves are shown to be the recommended testing material for the diagnosis of GFLV when sampling during the vegetative period, and phloem when sampling outside of the vegetative period.

GFLV infection reduces SWP in both of the cultivars ‘Schioppettino’ and ‘Refošk’, and when using both of the training systems (i.e., single and double Guyot systems) in the vineyard. For these different cultivars (‘Schioppettino’, ‘Refošk’) and using the different training systems (single and double Guyot systems), GFLV infection changed the expression of the RD22 ABA-responsive gene, the WRKY gene involved in ABA-responsive signalling networks, and the CHS2 and F3H1 genes involved in the anthocyanins metabolic pathway. No statistically significant differences in expression were seen for the NCED1, NCED2, SuSy, OLP, SAMT, F3H1 and LAR2 genes in the GFLV-infected grapevines compared to the healthy controls.

GFLV infection reduced SWP also under controlled conditions in a greenhouse for grapevines of the cultivar ‘Schioppettino’ grown in pots. Even greater difference between these healthy and GFLV-infected plants were observed for RHC. GFLV infection statistically significantly impacted upon genes involved in the ABA biosynthesis pathway (NCED1, NCED2), and on two genes
involved in anthocyanins synthesis (*CHS2, F3H1*). Under the influence of a water deficit lower than -0.7 MPa, *F3H2, RD22* and *WRKY* were statistically significantly up-regulated, and *LDOX* was down-regulated.

Additional, investigation of the GFLV presence in Northern Primorska also contributed to the general knowledge for winegrowers. Recognition of the GFLV symptoms and its diagnosis in vineyards are necessary to limit GFLV spread via vegetative propagation and/or throughout the vineyard (where *X. index* is present). Therefore, these data might have an impact on local grapevine production. Taking into account that GFLV infection can modify the physiology and gene expression of the grapevines also impacts on further studies on grapevines, which might indirectly impact on the international standards in future studies. Future studies will need to consider the specific interactions between symptoms development in grapevine and the effects of GFLV infection on locally and worldwide economically important grapevine cultivars. Moreover, as we showed here that GFLV infection reduces SWP and RHC, it will be interesting to further investigate xylem cavitation. Also, the role of GFLV on the target genes involved in drought adaption and on the whole plant metabolome will be the aims of additional studies.
7. SUMMARY

Grapevines (*Vitis vinifera* L.) represent one of the most important crops in the world in terms of both production and economic importance. Grapevines are exposed to many types of abiotic stresses (e.g., drought, flooding, low and high temperature, salinity) and biotic stresses (e.g., viruses, bacteria, phytoplasma, fungal disease) during their life-cycle. Therefore, grapevines elicit the appropriate defence mechanisms.

In the first part of this study, we monitored the occurrence of *Grapevine fanleaf virus* (GFLV) infection, which causes progressive decline of infected grapevines and lowers their yield. Grapevines were also tested for the presence of other viruses important for grapevines: *Arabis mosaic virus* (ArMV), *Grapevine leafroll associated virus* (GLRaV)-1,-2,-3,-4,-9, *Grapevine virus* A (GVA), *Grapevine fleck virus* (GFkV) in this study; and by Cigoj (2015): *Grapevine virus* B (GVB), *Tomato black ring virus* (TBRV), *Grapevine chrome mosaic virus* (GCMV), *Tomato ringspot virus* (ToRSV), *Raspberry ringspot virus* (RpRSV), *Strawberry latent ringspot virus* (SLRSV), and *Tobacco ringspot virus* (TRSV). Using ELISA, the presence of the following grapevine viruses were detected: GFLV, (GFkV), (GVA), and Grapevine leafroll associated viruses- 1,-2,-3,..

A wide range of GFLV symptoms caused by grapevine fanleaf disease in naturally infected vineyards were observed, including leaf, shoot and cluster malformations and leaf yellowing. GFLV is disseminated by its biological vector *X. index*, and through vegetative propagation of virus-infected material. The spread of GFLV in the vineyards was investigated here.

We constructed a spatio-temporal study of the GFLV titres during the seasons and throughout the grapevine, for its distribution in different grapevine organs through the season. This study shows that young leaves have high virus titres through the whole vegetative period, while mature leaves, tendrils and flower/ berry clusters only have high titres at the beginning of the vegetative period. The seeds retain high virus titres after berry colouring. Phloem scrapings were shown to contain lower virus titres during the vegetative period, with an increase outside and at the beginning of the
vegetative period. In flower/ berry clusters, mature leaves and tendrils, the GFLV titres decrease significantly over the vegetative period. Additionally, different GFLV titres were shown in five different cultivars, and different combinations of mixed infections with other grapevine viruses influenced the GFLV titre differently. Finally, correlation between the magnitude of symptom appearance and GFLV titres was analysed.

Grapevines adapt to abiotic stresses and biotic stresses by the expression of a wide range of stress-responsive genes, which are thought to have key roles in stress tolerance and survival.

SWP of the infected grapevines through the season was lower than SWP measured for healthy grapevines. For both seasons, there were significant differences in SWP measurements between healthy and GFLV-infected grapevines of ‘Schioppettino’ trained using the single Guyot training system.

SWP and RHC of the GFLV-infected grapevines were reduced compared to the healthy controls. The water deficit triggered the production of ABA, which induced the expression of the stress-related gene RD22. Additionally, this study shows that the WRKY gene that is involved in the ABA signalling network is regulated by water deficit. Plant defence responses to water stress also included up-regulation of the F3H2 and LDOX genes, which are involved in anthocyanins synthesis. GFLV infection significant impacted upon the expression of genes involves in ABA biosynthesis, as NCED1 and NCED2, and upon two genes involved in the early stages of anthocyanins synthesis, as CHS2 and F3H1. We also showed that the combination of grapevine cultivar, training system, and environmental conditions impacts on gene expression.

Key words: *Vitis vinifera* L., grapevine, *Grapevine fanleaf virus*; GFLV; grapevine disease; virus titre; distribution; fluctuation; ELISA; qPCR, ABA, drought, water status, water deficit, SWP, RHC, anthocyanins, gene expression
8. POVZETEK

BIOLOŠKA VLOGA VIRUSA PAHLJAČAVOSTI LISTOV VINSKE TRTE (GFLV) V VINORODNI DEŽELI PRIMORSKA

Vinska trta (Vitis vinifera L.) je ena izmed gospodarsko najpomembnejših kulturnih rastlin. Poleg abiotskih delavnikov, kot so suša, visoke in nizke temperature, poplave in zasoljenost tal jo pogosto ogrožajo, biotski delavniki (virusi, bakterije, fitoplazme in glive).

V sklopu študije z naslovom Biološka vloga virusa pahljačevosti listov vinske trte (GFLV) v vinorodni deželi Primorska smo morfološko pregledali trse v več izbranih vinogradih na Krasu, v Vipavski dolini in v Prepottu ter z laboratorijsko metodo ELISA testirali izbrane trse na viruse: GFLV, ArMV, Grapevine virus A (GVA), virus zvijanja listov vinske trte (Grapevine leafroll associated virus GLRaV od 1-9) in Grapevine fleck virus (GFkV). Grapevine virus B (GVB), Tomato black ring virus (TBRV), Grapevine chrome mosaic virus (GCMV) (Agritest test), Tomato ringspot virus (ToRSV), Raspberry ringspot virus (RpRSV), Strawberry latent ringspot virus (SLRSV) and Tobacco ringspot virus (TRSV) so bili testirani v študiji Cigoj, 2015. Poleg prisotnosti GFLV, smo potrdili okužbo z virusi: (GFkV), (GVA), and GLRaV-1, -2 in -3. Z ELISA testom smo na testiranih trsih odkrili okužbe z virusi GFLV, GFkV, GVA in virusi zvijanja listov (GLRaV).

V že obstoječem vinogradu, v katerem so prisotni z GFLV okuženi in zdravi trsi ter prenašalci GFLV, ogorčice X. index, smo analizirali bolezenska znamenja, ki jih povzroča GFLV. Ugotovili smo, da se virus v vinogradu prisoten in se v treh letih opazovan ni širil na sosednje ne okužene trse. Preučevali smo tudi pojav bolezenskih znamenj na sadilnem materialu, posajenem v obnovljen vinograd, v katerem so predhodno rastli z GFLV okuženi trsi in v katerem so bile prisotne ogorčice; X. index.

Za nadaljnje raziskave vpliva GFLV na pomembnejše agronomske parametre, kot so: število brstov, teža enoletnega prirasta in razmerje med pridelkom in prirastom enoletnega lesa, smo izbrali trse dveh lokalno pomembnih sort 'Refošk' in 'Pokalca' ('Schioppettino') brez okužbe na testirane viruse in pa trse, ki so okuženi z virusom GFLV.

Na izbranih trsih smo v dveh zaporednih letih merili vodni potencial trsov. Rezultati meritev so pokazali, da virusna okužba z GFLV vpliva na vodni status trsa. Vpliv virusa na trse je bil ovrednoten tudi s parametri teže lesa, dolžine med členkov, število brstov, številom grozdov. Analizirali smo tudi prisotnost/odsotnost povezave med virusi, ki okužujejo vinsko trto in izmerjenimi parametri grozdja in trsov. Rezultati so pokazali, da virusna okužba z GFLV vpliva na pomemben parameter rodnosti trsa t.j. teža prirasta enoletnega lesa.

Z metodo qPCR smo analizirali gene, ki so vključeni v biosintezo hormona abscizinske kisline (ABA), ki ima ključno vlogo pri odzivu rastline na sušo. Rezultati potrjujejo, da ima virus vpliv na izražanje genov v poti ABA. Analizirali smo tudi gene vključene v fenolno pot, v pot sladkorjev in v odziv rastline na biotski in abiotski stres, kjer se je pokazal vpliv virusa na izražanje nekaterih analiziranih genov.

Ključne besede: *Vitis vinifera* L., vinska trta, *Virus pahljačevosti listov vinske trte*; GFLV; bolezen vinske trte; količina virusa; razporeditev virusa; ELISA; qPCR, ABA, suša, vodni status, SWP, RHC, antocijani, ekspresija genov
Dedicated to my little shining star, Angel
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sisters, and my Mother-in-Law Ivanka and Father-in-Law Pavel, for being there for me. I also thank all my friends. Special thanks go to my dearest Vanja, Bojana, Martina and Maja, for being great friends on whom I can rely through every moment of my life. And finally I thank to all who help me with the thesis in any side of the view.
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Rules on wine (Ur.l. RS št. 111/13)


Taylor CR, Raski DJ, 1964. On the transmission of grape fanleaf by Xiphinema index. Nematologica, 10:489-4950


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**Supplementary Table 1:** Results of the two-ways ANOVA (considering infection and water stress as factors from the third analysis date on) for the grapevines planted in pots. The first two dates were processed with one-way ANOVA, as the water stress had not been applied yet.

<table>
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<tr>
<th>Status/Treatment</th>
<th>Time (days)</th>
<th>Gene</th>
<th>NCED1</th>
<th>NCED2</th>
<th>RD22</th>
<th>WRKY</th>
<th>SAMT</th>
<th>OLPI</th>
<th>SUSY</th>
<th>CHS2</th>
<th>F3H1</th>
<th>F3H2</th>
<th>LAR2</th>
<th>LDOX</th>
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**SUPPLEMENTARY**
Supplementary Table 2: Variability of component 1 (Factor 1) and component 2 (Factor 2) of the genes at days 0, 9 and 12 for the genes F3H1, F3H2, WRKY, SuSy, NCED1, NCED2, CHS2, SAMT, LAR2, RD22, OLP and LDOX. Variability >0.7 is considered as statistically significant.

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<th>Gene</th>
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Supplementary Table 3: P-values obtained through t-tests for the selected genes: $F3H1$, $F3H2$, $WRKY$, $SuSy$, $NCED1$, $NCED2$, $CHS2$, $SAMT$, $LAR2$, $RD22$ and $OLP$ for ‘Schioppettino’ and ‘Refošk’ grown in the field.

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