Journal of Plant Physiology 170 (2013) 121–128

Contents lists available at SciVerse ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Biochemistry

Antioxidative responses in *Vitis vinifera* infected by grapevine fanleaf virus

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ARTICLE INFO

Article history:
Received 17 April 2012
Received in revised form
10 September 2012
Accepted 10 September 2012

Keywords:
Antioxidative enzymes
Ascorbate
Glutathione
Hydrogen peroxide
Superoxide

ABSTRACT

The antioxidative response of grapevine leaves (*Vitis vinifera* cv. Trebbiano) affected by the presence of grapevine fanleaf virus was studied during the summer of 2010 at three different harvest times (July 1st and 26th, and August 30th). At the first and second harvest, infected leaves showed increases in the concentration of superoxide radical and hydrogen peroxide, the latter increasing for enhanced activity of superoxide dismutase. In contrast, at the last harvest time, increases in the ascorbate pool and ascorbate peroxidase activity maintained hydrogen peroxide to control levels. The glutathione pool was negatively affected as summer progressed, showing a decrease in its total and reduced form amounts. At the same time, increases in the ascorbate pool were observed, making antioxidant defenses of grapevine effective also at the last harvest time. Increases in phenolic acids, and in particular in p-hydroxybenzoic acid, at the first and second harvest might have enhanced the efficiency of the antioxidant system through an interrelation between a peroxidase/phenol/ascorbate system and the NADPH/glutathione/ascorbate cycle. The lack of increase in p-hydroxybenzoic acid at the third harvest could be due instead to the enhanced utilization of this acid for hydrogen peroxide detoxification. With time, grapevine plants lost their capacity to contrast the spread of grapevine fanleaf virus, but acquired a greater ability to counteract pathogen-induced oxidative stress, being endowed with more reduced antioxidant pools.

Introduction

Grapevine fanleaf virus (GFLV) causes fanleaf degeneration disease, which affects grape production worldwide. GFLV belongs to the *Nepovirus* genus of the *Comoviridae* family (Sanfacon et al., 2009) and is transmitted specifically by the soil-borne ectoparasitic nematode *Xiphinema index* and by infected planting material. GFLV is responsible for significant economic losses, reducing grape yield up to 80%, affecting fruit quality and shortening the longevity of grapevines (Andret-Link et al., 2004). Distorting virus strains elicit a wide range of symptoms in grapevine: shortening of internodes, zigzag growth and fasciation of shoots as well as asymmetry (resembling a fan) and acute indentation of blade leaves. In addition, the fruit set looks poor due to the presence of few and small bunches with aborted berries. Chromogenic strains of the virus induce various patterns of yellow discoloration (yellow mosaic form).

As grapevine is an agriculturally and economically important crop plant, its defense mechanisms against plant–pathogenic microorganisms have attracted considerable attention. Grapevines develop an active resistance response against pathogens like fungi and bacteria, and some resistance genes have been identified (Di Gaspero and Cipriani, 2002). Active defense mechanisms mainly involve accumulation of phenolics (constitutive or inducible), rapid and localized cell death, synthesis of pathogenesis-related proteins and of reactive oxygen species (ROS) produced in the plant oxidative burst (Bruno and Sparapano, 2006). These mechanisms do not act separately, but are often linked to each other in a chronological or consequential order. However, to date, no active resistance response against viral diseases has been found in grapevine (Espinoza et al., 2007).

Overproduction of ROS in the plant cell requires the intervention of antioxidant systems, which include metabolites such as ascorbate (AsA) and reduced glutathione (GSH) as well as scavenging enzymes, including superoxide dismutase (SOD) and ascorbate peroxidase (APX, Perl-Treves and Perl, 2002). In cell compartments including the apoplast (Sgherri et al., 2007), mixed systems such as the AsA–GSH cycle bring about the reductive detoxification of ROS at the ultimate expense of NAD(P)H (Wojtaszek, 1997). The production of ROS through an oxidative burst has been suggested to be the first line of defense against pathogen attack. The association of ROS formation and increased activity of enzymes participating in their metabolism with the induction of defense

Abbreviations: AsA, ascorbate; APX, ascorbate peroxidase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DIECA, N,N-diethyldithiocarbamic acid; GFLV, grapevine fanleaf virus; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; H, harvest time; H_2O_2, hydrogen peroxide; HR, hypersensitive response; NBT, nitroblue tetrazolium; $O_2^{\bullet-}$, superoxide radical; ROS, reactive oxygen species; SOD, superoxide dismutase.

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0176-1617/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.jplph.2012.09.016

responses has been demonstrated in both compatible and incompatible plant–pathogen interactions (Melillo et al., 2011; Mandal et al., 2011).

The activities of enzymes involved in the detoxification of ROS in the *Phaseolus vulgaris*-white clover mosaic virus compatible interaction were studied by Clarke et al. (2002). These authors suggested that virus replication and disease development were enhanced when the antioxidant enzymatic activities decreased. By contrast, induction of SOD, total peroxidase and APX activities was observed in the compatible interaction between cucumber mosaic virus and zucchini yellow mosaic virus with *Cucumis sativus* and *Cucurbita pepo* plants, respectively (Riedle-Bauer, 2000). In compatible interactions, multiple changes in gene expression, controlling the levels of virus in infected tissues, and an active role of hydrogen peroxide (H_2O_2), as well as of other ROS, in counteracting pathogen virulence have been suggested (Maule et al., 2002). Indeed, apart from being potential agents of oxidative stress, many studies have demonstrated that ROS play several essential roles in defense responses during plant–pathogen interactions, including as second messengers for the induction of defense response genes (Wang et al., 2010). Following avirulent pathogen attacks, ROS have been shown to participate in the signal transduction cascade(s) leading to defense reactions such as the hypersensitive response (HR) and systemic acquired resistance (Foyer and Noctor, 2003; Sgherri et al., 2007).

Little is known about the physiological consequences of GFLV infection. It has been suggested that the probable cause of decay of virus-infected vine plants might be not only the virus activity itself, but also reduced tolerance to repeated unfavorable environmental situations (Váradi et al., 2007). Indeed, GFLV disease causing fanleaf symptoms is accompanied by metabolic disturbances such as a decline in the photosynthetic performance and chloroplast degradation caused by elevated levels of ROS generated against the virus attack, which in turn may inhibit some key enzymes of photosynthetic CO_2 fixation (Christov et al., 2001; Váradi et al., 2007).

To date, the mechanisms involving the defense responses of grapevine against fanleaf disease have not been investigated in detail. The ability of plant cells to regulate the efficiency of their ROS-removal strategies is a key point in their resistance against pathogens. The aim of this study was to evaluate whether and how the antioxidant system of grapevine leaves was affected by the presence of GFLV and to assess whether changes over time in the plant defense system may be related to an increased resistance to the pathogen.

Materials and methods

Plant material

Two vineyards, a healthy control and a GFLV-affected vineyard were considered in this experiment. They were located at the Agro-Environmental Interdepartmental Research Center "E. Avanzi" of the University of Pisa (S. Piero a Grado) one km away each other. The vines (*Vitis vinifera* L. cv. Trebbiano) were grown under field conditions on a sub acid-sandy loam soil and were trained to a single curtain cordon. The infected vineyard has a long history of GFLV infection (10 years) and typical GFLV symptoms such as fanleaf distortion, shot berries and reduced yield were observed on the majority of vines. No readily observable differences were found among vines based on the severity and types of symptoms present. Infection of the selected material was ascertained by woody indexing on indicator species (*V. vinifera* cvs. Pinot noir and Chardonnay, and *Vitis berlandieri* × *Vitis riparia* Kober 5BB) and by Double Antibody Sandwich-Enzyme Linked Immunosorbent assay (DAS-ELISA) following the procedure of Clark and Adams (1977) modified by Boscia et al. (1991, 1995). The assay was carried out by coating Polysorp immunoplates (Nunc) with 200 μL per well of antibody solution (against GFLV) in carbonate buffer. Commercial kits produced by Agritest (Valenzano, Italy) were used. Alkaline phosphatase conjugates were added according to the manufacturer's instructions. Plates were read at 405 nm at 30 min intervals for 2 h in a BioTek (Winooski, VT, USA) automatic reader zeroed with an empty plate. Controls were included systematically and each sample was loaded in two different wells. Samples were considered positive when the mean absorbance was at least 3 SD units above the negative control. Leaf samples were collected from the marked tip of healthy and symptomatic shoots at three different harvest times (H) of summer 2010: July 1st (H1) and 26th (H2), and August 30th (H3). Soon after collection, samples were frozen in liquid N_2 and stored at −80 °C until analytical determinations.

Superoxide radical

In vivo detection of superoxide ($O_2^{\bullet}-$) was performed following the method of Sgherri et al. (2007) and slightly modified. For extraction of $O_2^{\bullet}-$, fresh leaves were immediately extracted at 4 °C with a solution consisting of ultrapure Milli Q water (Millipore, Milan, Italy), 73.4 μM nitroblue tetrazolium (NBT) and 10 mM N,N-diethyldithiocarbamic acid (DIECA). After centrifugation at 12,000 × *g* for 15 min, volumes of extracts were measured and the A_{560} quickly recorded. Calculations were performed using an extinction coefficient of 21.6 mM^{-1} cm^{-1}.

Hydrogen peroxide

The hydrogen peroxide (H_2O_2) concentration was evaluated following the method reported by Sgherri and Navari-Izzo (1995) based on the formation of the titanium-peroxide complex. Leaf tissue was homogenized at 4 °C with cold acetone and filtered. The precipitation of the complex was obtained by the addition of 5% titanyl sulphate and of concentrated NH_4OH solution to the extract. After centrifugation at 12,000 × *g* for 15 min, the supernatant was discarded and the pellet was washed with cold acetone. After 4 precipitations, the pellet was dissolved with 1.5 N H_2SO_4 and the solution was read at 415 nm. H_2O_2 content was calculated using a standard curve in the 0.5–10 μmol range.

Ascorbate (AsA) and dehydroascorbate (DHA)

Leaf tissue was homogenized in ice-cold 5% (w/v) trichloroacetic acid containing 4% (w/v) polyclar AT, using a cold mortar and pestle. AsA and total ascorbate (AsA + DHA) were determined in the supernatant following the method of Wang et al. (1991). Total ascorbate was determined through the reduction of DHA to AsA by 0.97 mM dithiothreitol, and DHA levels were estimated on the basis of the difference between total ascorbate and AsA contents. Two separate calibration curves for AsA and total ascorbate, covering the range of 5–50 nmol were used.

Reduced (GSH) and oxidized glutathione (GSSG)

Leaf tissue was homogenized at 4 °C in ice-cold 5% (w/v) trichloroacetic acid and centrifuged at 12,000 × *g* for 15 min. The supernatant was used for total (GSH + GSSG) and GSSG determinations by the 5,5'-dithio-bis-nitrobenzoic acid (DTNB)-GSSG reductase recycling procedure as reported by Sgherri and Navari-Izzo (1995). GSSG was determined after GSH had been removed by derivatization with 2-vinylpyridine. Changes in the absorbance of the reaction mixtures were detected at 412 nm at 25 °C. The amount of GSH was calculated by subtracting the GSSG amount, expressed as GSH equivalents, from the total glutathione amount.