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RESEARCH ARTICLE

Quercetin feeding protects plants against oxidative stress

[version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: Flavonoids are a complex group of plant-made phenolic compounds that are considered of high nutraceutical value. Their beneficial impacts on human health relate predominantly to their capacity to serve as antioxidants, thus protecting cells against the damaging impact of reactive oxygen species. Recent studies have also pointed at an essential role for flavonoids as antioxidants in plants.

Results: Here we show that the flavonoid quercetin, which is known to protect human cells from oxidative stress, has the same effect on plant cells. Under oxidative stress conditions, Arabidopsis plants grown on quercetin-supplemented media grew better than controls and contained less oxidized proteins. This protection was also observed in the dicot *Nicotiana tabacum* and the aquatic monocot *Lemna gibba*.

Conclusion: Quercetin can be used as a general antioxidant stress protectant for plants.

Keywords

Flavonoids, quercetin, antioxidants, Arabidopsis, tobacco, duckweed, paraquat

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Any reports and responses or comments on the article can be found at the end of the article.

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Introduction

Cellular redox homeostasis is maintained by a complex antioxidant defense system, which includes antioxidant enzymes and low-molecular-weight scavengers^{1–4}. Concerted action of the enzymatic and non-enzymatic components of this defense system counteracts excessive levels of reactive oxygen species (ROS), which can damage cellular components, while preserving adequate levels of ROS required for signaling and cellular redox regulation^{5–10}.

The rapid and excessive generation of ROS is a common response to abiotic stresses and thus can be viewed as a converging point for stress signaling and defense responses^{11–14}. One of the common responses to stress-induced ROS generation is increased flavonoid biosynthesis^{15–19}. Although there is a large body of evidence that supports a role for flavonoids as ROS scavengers, the actual *in vivo* function of flavonoids as antioxidants in plants was a matter of debate^{20–22}. The main points of contention were (1) that flavonoids are mainly found in vacuoles and are thus compartmentalized from the main site of ROS production in plant cells (*i.e.*, chloroplasts), (2) that flavonoids are enriched in epidermal cells and thus cannot play a significant role in protecting cells of the majority of plant tissues, and (3) that plant cells have an elaborate and efficient antioxidant defense system that successfully suppresses ROS accumulation and therefore the putative antioxidant role of flavonoids would be redundant²². However, recent studies both in *Arabidopsis* and other plant species have shown that the *in vivo* antioxidant function of flavonoids is important for the survival of plants under abiotic stress^{22,23}.

Recent studies have also shown that those flavonoid species which, based on their chemical structure, are predicted to be the strongest antioxidants are indeed induced the most by stress^{22,23}. These flavonoids, the dihydroxy B-ring-substituted flavonoids and their glycosides, are exemplified by quercetin and its derivatives²². Quercetin, one of the most abundant flavonoids in plants, also attracted significant attention in medical research because of its antioxidant, anti-inflammatory and anticancer effects with no human toxicity^{24,25}. Here we have tested if quercetin feeding protects plants against the ROS-inducer paraquat (methyl viologen). Paraquat causes the formation of ROS in plants predominantly by impacting the chloroplastic electron transport systems¹. Feeding *Arabidopsis*, tobacco and duckweed with quercetin indeed suppressed the toxic effects of paraquat, indicating that this flavonol can be used as an effective protectant against the harmful effects of ROS on plant growth.

Methods

Plant material and treatments

All plants were grown and treated under sterile conditions. *Arabidopsis* wild type Landsberg *erecta* (*Ler*) and *transparent testa* (*tt*) mutant lines *tt3-1*, *tt4-1* and *tt5-1* (all in *Ler* background) were grown on solid half-strength Murashige and Skoog (MS/2, Phytotechnology) media supplemented with 1% sucrose (pH 5.7). *Nicotiana tabacum* (Burley variety KT204LC) was grown on solid full-strength MS media with 3% sucrose (pH 5.7). *Lemna gibba* (Rutgers Duckweed Stock Cooperative ID 7749) was grown in liquid Schenk and Hilderbrandt Basal Salt media (SH, Phytotechnology) without sucrose and vitamins. All tested compounds were added to the media after autoclaving. Paraquat and quercetin were obtained from Sigma. Plants were grown in continuous light

with a light intensity of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 24°C. To measure fresh weight, at least seven pools of 10 plants per treatment were used. Chlorophyll levels were measured using CCM-300 chlorophyll content fluorometer (Opti-Sciences). Data were analyzed using Prism 5.0a software (GraphPad Software Inc.) and are presented as mean $\pm$ SD of at least two independent experiments. One-way ANOVA with the Bonferroni's multiple comparison post-test was used to determine the significance of the difference between means.

Protein isolation, derivatization and immunoblotting analyses

For the protein carbonylation experiments, plants were grown on the denoted media for 2 weeks and then weighed. Tissue was disrupted with zirconium beads in a BeadBug bead beater (MidSci) in 2 volumes of extraction buffer (50 mM potassium phosphate buffer pH 7.0, 2 mM MgCl_2 , 5% glycerol and 5 mM 2-mercaptoethanol). Protein concentration was measured with a BioPhotometer (Eppendorf) using Bradford reagent (Bio-Rad) and bovine serum albumin (BSA, Bio-Rad) as the standard. Proteins were derivatized as described previously²⁶. In brief, protein extracts containing the same amount of protein were mixed with one volume of 12% sodium dodecyl sulfate (SDS, Fisher Scientific) and 2 volumes of 20 mM dinitrophenylhydrazine (Sigma). Derivatization reactions were performed at room temperature in the dark for 60 minutes. Derivatization mixtures were then neutralized with 2M Tris Base and mixed with one volume of 2X SDS-PAGE loading buffer. Protein extracts used for the control gels were directly mixed with 1 volume of 2X SDS-PAGE loading buffer. After denaturation at 95°C for 5 minutes, protein samples were loaded onto SDS-PAGE gels (7.5% for derivatized proteins and 4–20% gradient for control proteins, both Mini-Protein TGX precast gels from Bio-Rad). Separated proteins were transferred to nitrocellulose membranes as previously described²⁷. The commercial antibodies used were rabbit polyclonal anti-DNP antibody (D9656 Sigma; used at 1:1000), monoclonal anti-HSP70 1D9 (Enzo; used at 1:10,000) and polyclonal anti-BiP antibodies (Santa Cruz Biotechnology, sc-33757; used at 1:1000). Secondary antibodies (goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP; Santa Cruz Biotechnology) were used at 1:1000. Immunoblots were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Signals were captured using ChemiDoc XRS (Bio-Rad).

Results

To determine whether quercetin feeding protects plants from oxidative stress, we tested the response of *Arabidopsis thaliana* wild type and mutants with reduced flavonoid content to the ROS-generating compound paraquat in the presence or absence of quercetin. Paraquat is known to prevent germination at high concentrations and to retard growth and promote chlorosis at sub-lethal concentrations^{28–30}. From the large collection of *Arabidopsis* flavonoid pathway mutants, we selected the three *transparent testa* (*tt*) mutants *tt3-1*, *tt4-1* and *tt5-1* in the *Ler* background^{31,32} and plated them on MS/2 media containing a range of paraquat doses (Figure 1A). The *tt4-1* mutant, which carries a lesion in the first dedicated enzyme of the flavonoid biosynthesis pathway, has been previously tested for paraquat sensitivity and was shown to have a lower tolerance to paraquat than the wild type by monitoring loss of chlorophyll content as a measure of chloroplast damage²³.

Figure 1. Quercetin suppresses paraquat toxicity in wild type and *tt* mutants. **A.** Seeds of the Arabidopsis wild type *Landsberg erecta* (*Ler*) and *transparent testa* (*tt*) mutants were sown and grown on half-strength Murashige and Skoog media containing the denoted compounds paraquat (PQ) and quercetin (Q). Representative plants were transferred to a new plate for photography 2 weeks after sowing. **B.** Relative fresh weight of plants grown on paraquat media with and without quercetin. Fresh weight of plants grown on control media was assigned the value of 1. Two-week-old plants were weighed in pools of 10 and the data are presented as mean $\pm$ SD (n $\geq$ 7).

Paraquat doses of 0.15 μ M and 0.3 μ M caused severe growth inhibition in both the wild type and *tt* mutants (Figure 1). Quercetin (at 100 μ M) alone did not lead to any significant changes in fresh weight of any of the tested lines (*Ler*: 73.9 $\pm$ 12.6 mg and 71.3 $\pm$ 10.1 mg; *tt4-1*: 73.2 $\pm$ 8.3 mg and 69.3 $\pm$ 8.7 mg; *tt5-1*: 68.8 $\pm$ 10.7 mg and 65.1 $\pm$ 6.7 mg; *tt3-1*: 66.2 $\pm$ 14.7 mg and 70.4 $\pm$ 8.4 mg for 2-week-old plants grown on control and 100 μ M quercetin supplemented media, respectively). When plants were grown on plates with 100 μ M quercetin and 0.15 μ M or 0.3 μ M paraquat, they were partially protected from the toxic effect of the herbicide (Figure 1). As expected from their genetic backgrounds, the wild-type, *tt3-1* and *tt5-1* seedlings were rescued more efficiently by 100 μ M quercetin than the *tt4-1* mutant which has the strongest defect in flavonoid biosynthesis (Figure 1).

To test if quercetin-dependent protection from oxidative stress can be detected at the molecular level, we analyzed protein oxidation. Protein carbonylation is an irreversible type of protein oxidation that leads to loss of protein function and is often used as an indicator of oxidative stress^{9,33–35}. We grew Arabidopsis wild-type plants on control plates and plates containing 100 μ M quercetin for 10 days. Plants were then harvested and incubated in either water or 100 μ M paraquat for 4 hours. Proteins were isolated, derivatized with dinitrophenylhydrazine, separated on SDS-PAGE gels, transferred to membranes and probed with the anti-diphenylhydrazine antibodies. The protective effect of quercetin was apparent from the reduced accumulation of derivatized proteins in paraquat-treated plants grown on media containing quercetin (Figure 2).

Figure 2. Quercetin feeding leads to reduced protein carbonylation in paraquat-treated plants. The wild-type plants (*Ler*) was grown for 10 days on control media or media supplemented with 100 μ M Q. Plants were then removed from the plates, weighed and incubated for 4 hours with a mock (water) or 100 μ M paraquat (PQ). Representative immunoblot of carbonylated proteins is shown. Arrowhead marks the position of the 50 kDa marker. HSP70 and BiP blots are shown to illustrate that the overall levels of proteostatic stress are not increased in the cytosol and endoplasmic reticulum, respectively. Region of the Ponceau S stained membrane encompassing the RuBiSCO large subunit (LSU) is shown as a loading control.

Next, we tested if quercetin protects other plant species from paraquat-induced oxidative stress. We chose to test tobacco as another dicot species that is distantly related to *Arabidopsis* and the aquatic monocot species *Lemna gibba* (duckweed) (Figure 3). Dose-response experiments showed that quercetin counteracts the toxic effects of paraquat in tobacco (Figure 3A and B). Whereas lower doses of quercetin (*e.g.* 10 μ M) did not reverse seedling growth inhibition or chlorophyll loss, seedlings grown on paraquat and higher quercetin doses (*e.g.* 50 μ M and 100 μ M) showed no symptoms of toxicity. Seedlings grown on paraquat and the highest tested dose of quercetin (500 μ M) remained green, but were stunted suggesting that quercetin concentrations higher than 100 μ M are suboptimal for tobacco growth.

We also observed a protective effect of quercetin against paraquat toxicity in the duckweed *Lemna gibba*. Duckweeds are the smallest, fastest growing and the most morphologically reduced flowering plants^{36,37}. They have a frond (thalloid), no stem and one or more roots. When duckweed plantlets were grown for 36 hours in liquid media with 1 μ M paraquat, new fronds emerged as chlorotic (Figure 3C). In contrast, new-grown fronds remained green when plantlets are grown in media containing 1 μ M paraquat and 100 μ M quercetin. Chlorophyll measurements showed that the overall chlorophyll level in paraquat-treated cultures decreased to ~50% of the control, whereas the chlorophyll level in cultures treated with paraquat and quercetin were the same as in the control plants (Figure 3D).

Figure 3. Quercetin rescues tobacco and duckweed from paraquat toxicity. **A.** Tobacco KT204LC seeds were sown and grown on full-strength Murashige and Skoog media containing the denoted doses of PQ and/or Q. Plants were grown for 3 weeks prior to photography. Scale bar: 5 mm. **B.** Plants shown in **A.** were used to measure chlorophyll (Chl) content. One-way ANOVA was used to determine the significance of the difference between the PQ-treated sample and PQ and Q-treated sample. ***, $p < 0.001$. **C.** *Lemna gibba* plantlets were transferred to Schenk and Hilderbrandt media with denoted doses of PQ and/or Q and incubated under continuous light with the denoted doses of PQ and/or Q for 5 days prior to photography. Arrowhead points to the newly grown fronds. Scale bar: 5 mm. **D.** Chl content was measured in all (young and mature) fronds from plantlets shown in **C.** One-way ANOVA was used to determine the significance of the difference between samples. ***, $p < 0.001$.

Dataset 1. Raw data of quercetin feeding protecting plants against oxidative stress

<http://dx.doi.org/10.5256/f1000research.9659.d136964>

The raw data used to develop the graphs in [Figure 1](#) and [Figure 3](#) are available as three separate files.

Conclusion

Here we have shown that feeding plants with quercetin suppressed paraquat toxicity, indicating that this particular flavonoid and its derivatives have an important role in the protection of plant cells against increased ROS load. We also found that quercetin offers protection against ROS in a range of plant species, from the evolutionary distant dicots *Arabidopsis* and tobacco to the monocot and aquatic plant *Lemna gibba*. Thus, we can conclude that quercetin can be used as a general stress protectant. Considering the relatively low cost of quercetin and its low concentration (100 μ M) required for protection against ROS, we propose that the inclusion of quercetin to growth media could be beneficial to promote stress tolerance of agricultural plants grown in tissue or aquaculture.

Data availability

F1000Research: Dataset 1. Raw data of quercetin feeding protecting plants against oxidative stress, [10.5256/f1000research.9659.d136964](https://doi.org/10.5256/f1000research.9659.d136964)³⁸

Author contributions

J.K., T.E.S. and J.A.S. designed the experiments. J.K. and T.E.S. performed the experiments. All authors analyzed the data, prepared figures and wrote the paper.

Competing interests

Authors declare no competing interest.

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Frank Van Breusegem

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This concise manuscript provides clear evidence that addition of quercetin in growth medium protects plants against Methyl Viologen stress (as monitored by growth phenotypes and decreased protein carbonylation) and that quercetin can alleviate stress sensitivity in flavonoid biosynthesis Arabidopsis mutants. The conclusions are sensible and justified on the basis of the described results.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 10 October 2016

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Keith R. Davis

Biotechnology Program, Johnson Center for Innovation and Translational Research, Indiana University, Bloomington, IN, USA

This manuscript provides solid data indicating that quercetin can protect plants grown in tissue culture or aquaculture conditions from the oxidative stress imposed by paraquat, which interferes with chloroplast electron transport. The experiments are adequately described and scientifically valid, albeit, they are limited to basic assays of plant growth and overall protein oxidation levels.

The data shown appear to be of good quality and appropriate statistical analyses have been provided in most cases. The major weaknesses of the manuscript are that the studies are rather preliminary and the major general conclusions are not entirely supported by the data. The manuscript would be strengthened if the authors addressed the following points by providing more data or limiting the conclusions to what is clearly supported by the data:

1. The conclusion that quercetin can be used as a general antioxidant stress protectant for plants is too broad and would require additional data to be accurate. First, only one type of oxidative stress was assessed; additional assays compatible with the tissue culture format (e.g. salt, metal, cold stress etc.) are needed to support this generalization. Second, the authors only point out at the end of the Discussion that the use of quercetin as a stress protectant is likely limited to tissue culture or aquaculture of plants. This point should be made clear earlier in the manuscript and in the abstract.
2. It isn't clear how quercetin exerts the observed effects. The authors appear to believe that it is due to the well documented antioxidant properties of this flavonoid; however, no data are presented to indicate whether quercetin was taken up by the plants and if so, how the amounts accumulated compared to what is normally found in the test plant species. It is well documented in animal cells that quercetin affects a number of signaling pathways (e.g. JNK and other MAP kinases, Akt etc.) that are conserved in plants. Thus, it is possible that quercetin-induced tolerance may be more complex than simply scavenging ROS.

Several minor points that would be helpful to clarify are:

1. What treatments summarized in Figure 1 are statistically significantly different? The authors suggest that the rescue of *tt4* plants was not as robust as that observed in wild-type or the other *tt* mutants tested; however, based on the error bars, it isn't clear how significant the observed trend is.
2. Whether the reduction in protein oxidation observed in the acute short-term treatments also occurred in plants treated under the conditions of the growth assays.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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