BIOLOGIA PLANTARUM 59 (3): 554-559, 2015 DOI: 10.1007/s10535-015-0508-z

Europium improves the transport of quercetin through *Arabidopsis thaliana*

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Abstract

The effect of a rare earth element europium (Eu) on the long-distance transport of a plant defence compound quercetin (Q) was investigated. The complex Q/Eu^{3+} was synthesized in a HEPES buffer and tested for its transport ability through *Arabidopsis thaliana* and its ability to interact with target molecules in plant cells. Our results show that complexation with Eu^{3+} enhanced the transport of Q through *Arabidopsis* roots. During the transport, the complex degraded and released a free Q to tissues where Q was originally not available. Thus, the plant became better supplied with the defensive compound Q. The obtained spectrophotometric data imply that one of the reasons for the Q/Eu^{3+} degradation was the interaction of the complex with double stranded RNAs (dsRNAs) present in *Arabidopsis*. Since dsRNAs are replicative forms of plant RNA viruses, the ability of Q/Eu^{3+} to release a free Q in their presence suggests further investigation of this complex as a potential antiviral agent.

Additional key words: antiviral agents, long-distance movement, polyphenols, Q/Eu^{3+} complex.

Introduction

Europium (Eu) is one of the more abundant rare-earth elements. It does not possess any known environmental threat to plants or animals and, according to Emsley (2003), can be found in the 30 - 130 ng g^{-1}(d.m.) range in some plants. The Eu^{3+} can be transported through a plant (Fellows *et al.* 2003) and may play important roles in its physiology (Gao *et al.* 2003). A treatment with Eu^{3+} affects the distribution and related biological activities of elements in *Lathyrus sativus* roots (Tian *et al.* 2003). The Eu^{3+} also activates proteolytic enzymes and elevates the activity of Na^+/K^+-ATPase by changing the ratio of Na^+/K^+ (Tian *et al.* 2005). Zeng *et al.* (2003) proposed that Eu^{3+} can play an important role in plant development by promoting Ca^{2+} transport across the plasma membrane.

Flavonoids are a group of plant polyphenols indispensable for normal functioning and survival of plants in the environment. In plants, flavonoids act as strong antioxidants (Ahmed *et al.* 2009, Quideau *et al.* 2011), are involved in flower coloration (Chen *et al.* 2012), affect plant growth and development (Jacobs and Rubery 1988, Levizou *et al.* 2004), affect auxin distribution (Rusak *et al.* 2010), exert antiviral and antifungal activities (Rusak *et al.* 1997, Ahn *et al.* 2014), and act as signal molecules. Moreover, they have been described as a novel class of hormones (Stafford 1991). Nevertheless, there is still one intriguing fact about them; even though they exert a plethora of positive biological effects in plants, their ability of long-distance movement through a plant is quite limited. Furthermore, Buer *et al.* (2007) confirmed that, when exogenously applied to different parts of *Arabidopsis*, flavonoid quercetin (Q) is taken up only at root tips and its mobility is minimal.

Flavonoid/metal complexes are considerably more effective radical scavengers than free flavonoids (Kostyuk *et al.* 2001, 2004). González-Álvarez *et al.* (2005) demonstrated that Cu^{2+} complexes of certain flavonoids are more active as nucleases than the parent flavonoids. Rusak *et al.* (2009) revealed that the complex of Q with lanthanum (La^{3+}) has a significantly higher

Submitted 23 July 2014, *last revision* 23 October 2014, *accepted* 1 December 2014.
Abbreviations: CMVsat - satellite-associated *Cucumber mosaic virus*; DPBA - diphenylboric acid-2 aminoethyl ester; dsRNA - double-stranded RNA; Eu - europium; HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Q - quercetin; ssRNA - single-stranded RNA.
Acknowledgements: This work was financially supported by the Ministry of Science, Education and Sport of Croatia (098-0982914-2918 and broj Rusak projekta) The authors are grateful to Dr. M. Hadžija, the Laboratory for Molecular Endocrinology and Transplantation, Ruđer Bošković Institute, for help with a fluorescence microscope.
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affinity towards a viral satellite double stranded RNA than Q or La^{3+} alone. Ward *et al.* (1981) showed that the halogenation of some flavonoids increases their activity against *Staphylococcus aureus*, whereas Wang *et al.* (1992) reported that the binding of transition metals to some flavonoids also increases their antibacterial activities. Obviously, the derivatization of flavonoids facilitates their interactions with target biomolecule(s) or conjugation to a read-out tag (Carlson 2010). The previously mentioned effects of the flavonoid/metal complexes are gained *in vitro*, and to our knowledge until now, there have been no published data on flavonoid/metal complexes effects *in planta*. Moreover, the Q/Eu^{3+} as well as other flavonoid/Eu^{3+} complexes has so far been synthesized only in non-biological buffers (Woźnicka *et al.* 2007), and there are no data about their synthesis in biologically relevant buffers.

Therefore, taking into account the fact that the binding of a metal to a flavonoid positively changes the properties of the latter (Kostyuk *et al.* 2001, 2004, González-Álvarez *et al.* 2005), that a rare-earth element binding enhances the affinity of Q towards viral double stranded RNAs (Rusak *et al.* 2009), and that Eu^{3+} itself is able to affect the physiological processes in plants (Gao *et al.* 2003, Tian *et al.* 2003, 2005, Zeng *et al.* 2003), we wanted to test *1*) whether Eu^{3+} can affect a long-distance transport ability of Q through a plant, and *2*) whether (and how) Eu^{3+} can affect the interaction of Q with RNA molecules present in *Arabidopsis*. We chose Q as model flavonoid since it is the most abundant and, so far, the best described flavonoid (Dolatabadi 2011) with many positive biological effects in different model organisms (Erlund 2004).

Materials and methods

Arabidopsis thaliana Heynh. ecotype Columbia (Col-0) seeds were purchased from the Nottingham Arabidopsis stock centre (NASC, Nottingham, UK). The seeds were synchronized on a wet filter paper at 4 °C for 3 d, sterilized with a 2.55 % (m/v) aqueous solution of *Izosan* (commercial bleach), and transferred to a Murashige and Skoog basal growth medium. Plates were maintained in a controlled growth chamber at a temperature of 23 ± 1 °C, a 16-h photoperiod, and an irradiance of 100 μmol m^{-2} s^{-1}. Three-week-old seedlings were used for a transport experiment.

The formation of a quercetin/europium (Q/Eu^{3+}) complex in a biological HEPES buffer (100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.0) was monitored using a *Varian Cary 100 Bio UV/Vis* spectrophotometer (*Varian*, Palo Alto, CA, USA). A stock solution of Q was prepared at a 10 mM concentration in an absolute ethanol, and prior to the measurement diluted with the HEPES buffer to a concentration of 10 μM. An aqueous stock solution of Eu^{3+} was prepared at a 10 mM concentration, and small aliquots were successively added to the buffered aqueous solution of Q. Obtained data were analysed using the *Origin6.1* software.

Arabidopsis seedlings were incubated in the 100 mM HEPES buffer to which either Q at a final concentration of 10 μM, or the Q/Eu^{3+} complex at a final concentration of 10 μM/55 μM (the ratio according to the results of UV/Vis titrations) was added. The stock solution of Q in the absolute ethanol was 10 mM, so the total percentage of ethanol was 0.1 % (v/v), which proved to be non-toxic for the seedlings. The incubation lasted 5 h after which the seedlings were thoroughly washed with water and subsequently stained with 0.25 % (m/v) diphenylboric acid-2 aminoethyl ester (DPBA) and 0.005 % (v/v) *Triton X-100* for 30 min (adapted from Buer and Muday 2004). After staining, the seedlings were washed with a 0.5 mM aqueous solution of $CaCl_2$ and then with water. Fluorescence was visualized under an *Olympus* (Singapore) *BX51* fluorescence microscope equipped with a *DP50* camera at a 10× magnification using the *Viewfinder Lite 1.0* image acquisition software (*Better Light*, San Carlos, CA, USA). The fluorescence emission was visualized after excitation using a spectral filter with the range of 460 - 490 nm.

The plants were infected with satellite-associated *Cucumber mosaic virus* (CMVsat) which was reported for the first time in Croatia by Škorić *et al.* (1996); this virus is deposited in the Laboratory for Virology, Department of Microbiology, Faculty of Science, University of Zagreb under the label 14A. The procedure of double-stranded RNAs (dsRNAs) isolation from the CMVsat-infected plants was performed as described by Kearney *et al.* (1990) with small modifications. A total nucleic acids extract was adjusted to 16 % (v/v) ethanol and applied to a *CF-11* cellulose column pre-prepared in an Eppendorf tube [50 mg of cellulose powder was mixed with 0.2 cm^3 of a 16 % ethanol-adjusted STE buffer (0.1 M NaCl, 0.05 M Tris, 1 mM Na_2EDTA, pH 6.8)]. The cellulose with bound dsRNAs and the solution with single-stranded RNAs (ssRNAs) were separated by centrifugation (5 000 *g*, 4 °C, 6 min). Further, three washes (consisting of vortexing in 0.4 cm^3 of 16 % ethanol-adjusted STE buffer for 15 s) of the dsRNAs-bound cellulose and centrifugation (5 000 *g*, 4 °C, 4 min) were performed. The dsRNAs bound to the cellulose were eluted by vortexing in 0.25 cm^3 of a pre-cooled ethanol-free STE buffer for 1 min, and centrifuging (5 000 *g*, 4 °C, 10 min). The precipitation of RNAs with a 1/10 volume of sodium acetate (3 M, pH 5.5) and 4 volumes of a cold ethanol (-20 °C) was followed overnight. The RNAs were collected by centrifugation (10 000 *g*, 4 °C, 30 min), re-suspended in a TE buffer (10 mM Tris, 1 mM Na_2EDTA, pH 7.5), and stored at -20 °C. The same procedure was carried out for the isolation of RNAs from healthy plants.

The interactions of the Q/Eu^{3+} complex with RNA in a HEPES buffer were monitored using the *Varian Cary 100 Bio* UV/Vis spectrophotometer. The Q/Eu^{3+} complex was prepared as described in the chapter above and aliquots of RNAs were successively added to the buffered aqueous solution of the complex.

Results

The formation of Q/Eu^{3+} complex was studied in a biological HEPES buffer with the aim of applying the complex *in vivo*. The UV/Vis spectrum of Q revealed the typical absorption maxima at 250 and 370 nm (Fig. 1*A*), the former related to a benzoyl chromophore and the latter to a cinnamoyl chromophore (Marinić *et al.* 2006). Successive additions of the aliquots of the Eu^{3+} stock solution to the buffered aqueous solution of Q (10 μM) yielded considerable changes in the Q UV/Vis spectrum (Fig. 1*A*). The absorbance peak at 370 nm gradually decreased, and a new peak at 446 nm appeared (Fig. 1*B*). This peak corresponds to a newly formed complex Q/Eu^{3+}. A more pronounced change in the cinnamoyl part of the Q absorbance spectrum indicates a stronger involvement of the ring B in the formed complex.

Titration data (changes in complete spectra) were processed by the multivariate non-linear least-square fitting analysis of the *Specfit* program (Maeder and Zuberbuehler 1990). The best fit between the experimental and the calculated data was found for the 1:1 stoichiometry of $Q:Eu^{3+}$ (data not shown). The result of the UV/Vis titration indicates the five times excess of Eu^{3+} over Q was necessary for the formation of the Q/Eu^{3+} complex (Fig. 1*B*).

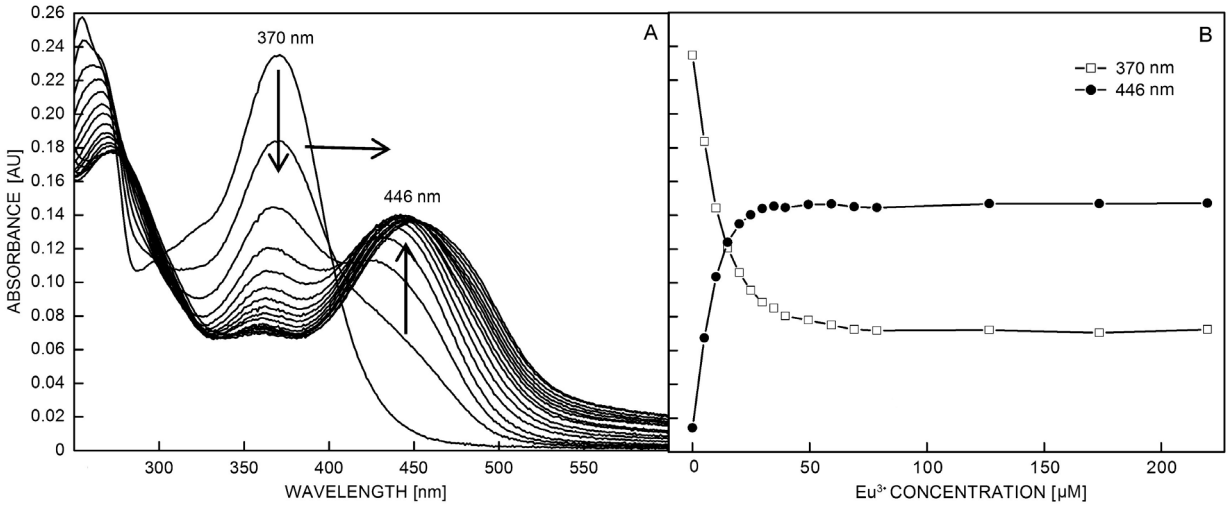

Fig. 1. *A* - Changes in UV/Vis spectra of Q (10 μM) upon titration with Eu^{3+} (5 - 225 μM). The spectra are corrected for the dilution and contribution of Eu^{3+} absorption. *B* - Changes of the absorption maximum of Q (10 μM) at 370 nm and of a new absorption maximum at 446 nm attributed by *Specfit* processing of titration data (here not shown) to the Q/Eu^{3+} complex as function of different concentrations of added Eu^{3+}. All the measurements were performed in a HEPES buffer (100 mM, pH 7.0).

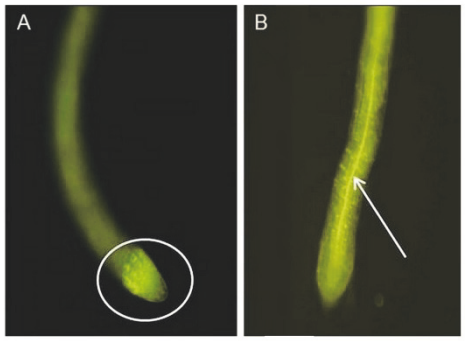

Fig. 2. Roots from CMVsat-infected *Arabidopsis thaliana* incubated in the free Q (*A*) or Q/Eu^{3+} complex (*B*). The plants were treated with a flavonoid specific dye DPBA and imaged by a fluorescence microscope with a 10× magnification. The preparations were scanned using a spectral filter *BP 460-490 nm*; *LP 515-800 nm*. The *oval* in *A* denotes the position of the exogenously applied free Q and the *arrow* in *B* denotes the position of the Q/Eu^{3+} complex in the root. The experiment was repeated with seven independent biological replicates with similar results.

Metal complexation significantly altered not only the electronic properties of Q (Fig. 1) but also significantly decreased the hydrophobic nature of Q and introduced a net positive charge (3+) on the otherwise neutral flavonoid molecule. The latter two changes could have a significant impact on the mobility of Q through plant including the root absorption. As proof-of-principle, we performed a set of experiments on the *Arabidopsis* (Col-0) seedlings, testing the absorption efficiency of Q or the Q/Eu^{3+} complex from the surrounding solution, as well as the Q distribution along the seedlings. The complexation of Q with Eu^{3+} was carried out *in vitro* in a biological HEPES buffer, and the complex Q/Eu^{3+} or free

Q as control was applied to *Arabidopsis* roots. After incubation, the roots were stained with DPBA, and Q or Q/Eu^{3+} were visualized under a fluorescence microscope.

As shown in Fig. 2, Q stopped at the place where it was originally taken up, at the root tip. Conversely, the Q/Eu^{3+} complex was transported a considerably longer distance *via* the central root vein. Moreover, a fine filament distribution stemming from the central vein was also evident. This result indicates that the long-distance transport of Q could be stimulated by a metal complexation. During the transport, the complex degraded and released free Q to tissues where originally it was not available.

Successive additions of small aliquots of dsRNAs, isolated from *Arabidopsis* infected with CMVsat, to the buffered aqueous solution of the Q/Eu^{3+} complex resulted in significant changes in the UV/Vis spectrum of the complex (Fig. 3*A*). Upon the addition of dsRNAs, the hypochromic change of the Q/Eu^{3+} absorption maximum (λ = 446 nm) occurred, accompanied by a simultaneous absorption increase at 370 nm, resulting in the UV/Vis spectrum corresponding to the free Q at the end of the titration (Fig. 3*B*). This suggests a dsRNA-induced degradation of the Q/Eu^{3+} complex. Considering the long-distance transport issue, this result indicates that dsRNAs were one of the molecular structures present in CMVsat-infected *Arabidopsis* that were responsible for the Q/Eu^{3+} complex degradation and release of a free Q. On the other hand, in the presence of ssRNAs, a free Q was not released from the complex Q/Eu^{3+} (Fig. 4*A*,*B*).

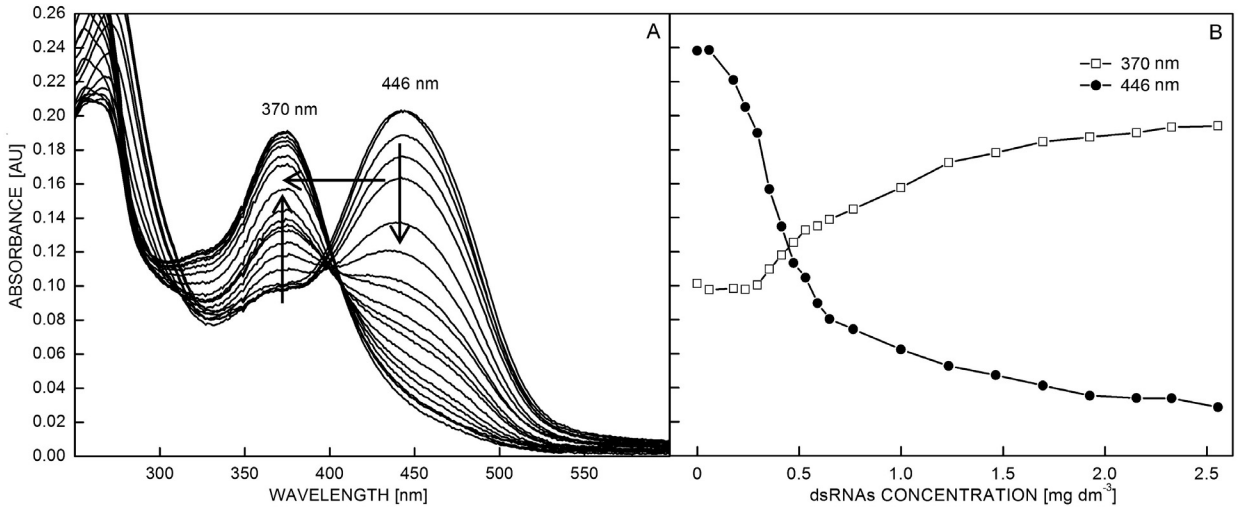

Fig. 3. *A* - Changes in UV/Vis spectra of the Q/Eu^{3+} complex (10 μM Q) upon titration with dsRNA (0.06 - 2.63 mg dm^{-3}) isolated from CMVsat-infected *Arabidopsis*. The spectra are corrected for dilution and contribution of dsRNA absorption. *B* - Changes of the absorption maximum of Q at 370 nm and of the Q/Eu^{3+} complex at 446 nm as function of different concentrations of added dsRNA. All the measurements were performed in a HEPES buffer (100 mM, pH 7.0).

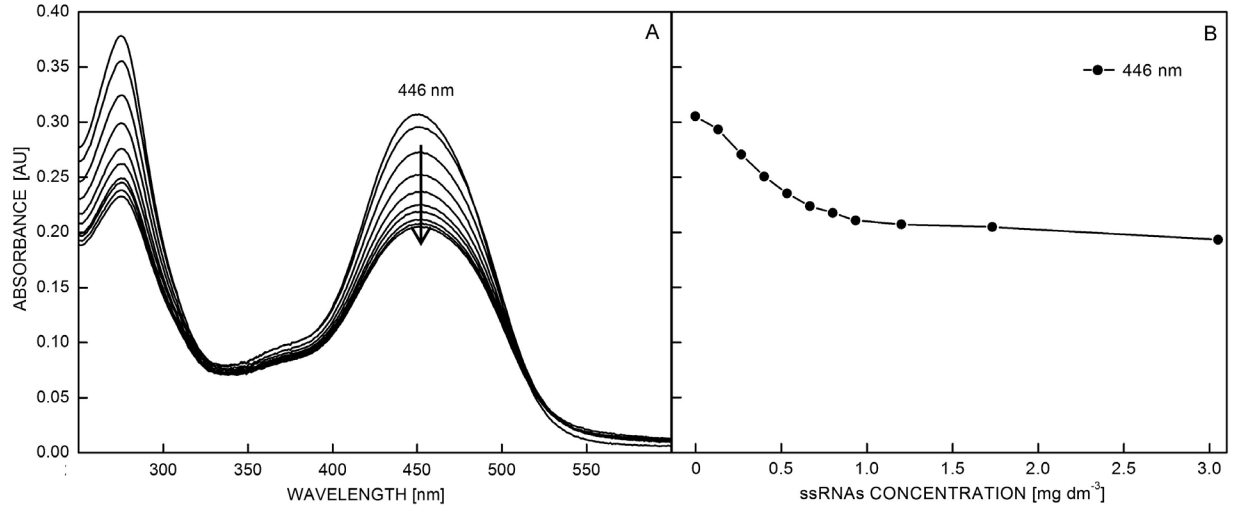

Fig. 4. *A* - Changes in UV/Vis spectra of the Q/Eu^{3+} complex upon titration with ssRNA (0.06 - 3.05 mg dm^{-3}) isolated from CMVsat-infected *Arabidopsis*. The spectra are corrected for dilution and contribution of ssRNA absorption. *B* - Changes of the absorption maximum of the Q/Eu^{3+} complex as function of different concentrations of added ssRNA. All the measurements were performed in a HEPES buffer (100 mM, pH 7.0).

Discussion

Improved biological effects of flavonoid/metal complexes in comparison to those of free flavonoids attract much attention (Kostyuk *et al.* 2001, 2004, González-Álvarez *et al.* 2005, Rusak *et al.* 2009). The evidence that the rare-earth metal Eu is present in some plants (Emsley 2003), is able to be transported within plants (Fellows *et al.* 2003), and promotes the transport of certain ions across the plasma membrane (Zeng *et al.* 2003) encouraged us to test whether this metal could affect the long-distance transport ability of the defensive compound Q. For that purpose, we first formed the Q/Eu^{3+} complex in a biological HEPES buffer. The property of Eu and Eu-based complexes to show a fluorescence emission (Leif *et al.* 2006) as well as the fact that Q and Q-based complexes in combination with the flavonoid specific dye DPBA emit fluorescence supported the use of a fluorescence microscope in monitoring the Q/Eu^{3+} long-distance transport through the root. The results presented here reveal that the applied Q remained where it was originally taken up (at the root tip), however, the complex Q/Eu^{3+} was transported to a longer distance. Moreover, the fluorescence microscopy results clearly show that the transport of the Q/Eu^{3+} complex was through the root central vein. Obviously, Eu^{3+} could direct Q from the *Arabidopsis* root tip into the root central vein, and subsequently stimulated the long-distance transport of Q through the *Arabidopsis* root. Even if Q itself has a very limited long-distance transportation ability, it still positively affects plant defence responses against viruses (French and Towers 1992, Malhotra *et al.* 1996, Rusak *et al.* 2007). Thus, its improved long-distance transport can lead to a better protection of plant tissues. To the best of our knowledge, this is the first report on the chemical improvement of a flavonoid long-distance transport through a plant.

According to the findings of Buer *et al.* (2007) regarding flavonoids naringenin, dihydrokaempferol, and dihydroquercetin being symplastically long-distance transported through *Arabidopsis*, we presume that the transport of the Q/Eu^{3+} complex through the *Arabidopsis* root could also use a similar pathway. Moreover, the induced long-distance transport of Q/Eu^{3+} could be due to the improved interaction of the complex with certain membrane transporters in comparison to Q alone. This is in accordance with the finding that Eu^{3+} can elevate the activity of Na^+/K^+-ATPase by changing the Na^+/K^+ ratio (Tian *et al.* 2005). An increased ATP-ase activity would stimulate flavonoid translocation since ATP-dependent ABC-type transporters are known to be largely involved in the flavonoid transport (Frangne *et al.* 2002).

So far, it has been shown that a plant-specific metabolite nicotianamine is able to form stable complexes with different metals (Benes *et al.* 1983, Anderegg and Ripperger 1989) and the transport of them through roots *via* the xylem sap (Curie *et al.* 2009). Here we show, on the example of a flavonoid, the existence of an opposite effect – the metal Eu^{3+} supported transport of the flavonoid Q through a plant root.

The fact that the Q/Eu^{3+} complex was degraded and released a free Q during the transport suggests its possible interaction with certain compounds present in *Arabidopsis*. Since Rusak *et al.* (2009) previously proved that the rare-earth metal lanthanum significantly enhances the ability of Q to bind to a viral satellite dsRNA, we presumed that Eu^{3+} could also enhance the interaction of Q/Eu^{3+} with RNA present in virus-infected *Arabidopsis*. Therefore, we examined the interactions of this complex with ssRNA and dsRNA isolated from *Arabidopsis* infected with CMVsat using a UV/Vis spectrophotometry. The results reveal that the Q/Eu^{3+} complex degraded and released a free Q in an interaction with dsRNAs, whereas remained stable in an interaction with ssRNAs. This is interesting if we know that plants infected with RNA viruses readily contain detectable amounts of viral dsRNAs ($> 0.1 \times 10^6$) (Dodds *et al.* 1984), which represent viral replicative forms and opens up the possibility of exploring Q/Eu^{3+} as a potential antiviral agent. Namely, in the presence of viral dsRNAs, Q/Eu^{3+} degrades into a free Q that could further act against viruses (Malhotra *et al.* 1996, Rusak *et al.* 2007, Krcatović *et al.* 2008).

An ability to enhance the long-distance transport of Q through a plant and its interaction with viral replicative intermediates (dsRNAs) could have a tremendous impact on a better protection against viruses for the whole plant. Our results suggest a new biological role of the rare-earth metal Eu^{3+} as well as a new approach to the long-distance transport of Q in plants.

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