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Effect of flavonoids on heavy metal tolerance in *Arabidopsis thaliana* seedlings

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ABSTRACT. *Arabidopsis thaliana* was used to investigate possible effects of flavonoids on heavy metal tolerance. *Arabidopsis* wild type (WT) and mutant lines with a defect in flavonoid biosynthesis (*tt*) were grown on media containing different heavy metals, and two growth parameters were evaluated. It was shown that root length and seedling weight were reduced in mutants more than in the wild type when grown on cadmium (Cd) while on zinc (Zn) only root length was affected. As marker for heavy metal tolerance, the induction of two phytochelatin synthase genes (*AtPCS1*, *AtPCS2*) in WT and the *tt5* mutant after Cd treatment was monitored by quantitative RT-PCR. The *AtPCS1* transcript was induced in WT at higher Cd levels than in the mutant. In contrast, the *AtPCS2* transcript was not induced under these experimental conditions in the WT, but it was induced in *tt5*. We then investigated whether the growth inhibition phenotype of the mutants could be rescued by addition of flavonoids. For this, the seedlings were treated with the flavanone naringenin and the flavonol quercetin, and the same growth parameters were determined. Quercetin and naringenin induced root growth and seedling fresh weight of WT on concentrations of Zn up to 500 μM, but this was not the case for root length of *tt* mutants. However, seedling weight could be stimulated by flavonoids, especially in the *tt5* mutant line. When the plants were grown on Cd, only the root length defect of *tt5* plants could be rescued by flavonoids. The seedling weight increased on Cd in all lines, although to a different extent. These results are discussed with respect to the potential role of flavonoids in heavy metal tolerance.

Keywords: Antioxidant; *Arabidopsis thaliana*; Heavy metal tolerance; Flavonoids; Phytochelatin synthase; *Transparent testa* mutants.

INTRODUCTION

As a consequence of industrial development, the environment is increasingly polluted with heavy metals (Michalak, 2006). Plants possess homeostatic mechanisms that allow them to keep essential metal ions in cellular compartments and minimize the damaging effects of an excess of nonessential ones. One of the adverse effects heavy metals have on plants is the generation of harmful active oxygen species, leading to oxidative stress. Besides the well-studied antioxidant systems consisting of low-molecular antioxidants and specific enzymes, recent works have begun to highlight the potential role of flavonoids, phenylpropanoids, and phenolic acids as effective antioxidants (Michalak, 2006). Flavonoids are secondary plant metabolites with a vast array of possible functions, including antioxidative activity (Brown et al., 1998; Havsteen, 2002). The functional diversity of flavonoids is due to their structural diversity, and to date more than 10,000 different molecules are known. This diversity endows flavonoids with many more biological functions in addition to their roles as antioxidants in the plant (Tahara, 2007).

Besides being radical scavengers, flavonoids are able to function as chelators for metals, depending on the molecular structure (Brown et al., 1998; Aherne and O'Brien, 2000; Soczynska-Kordala et al., 2001; Michalak, 2006; Korkina, 2007). In addition, anthocyanins, which are synthesized by the same route as flavonoids, have been implicated in tolerance to stressors such as drought, UV-B, and heavy metals (Gould, 2004). In the fungus *Alternaria alternata* the addition of quercetin and morin conferred substantial protection against the inhibition of fungal growth by copper while naringenin and rutin were less effective (Park et al., 1999). For cell cultures of *Ginkgo biloba* it was demonstrated that flavonoids accumulated in answer to heavy metal stress up to 12-fold in response to $CuSO_4$ treatment as compared to that of untreated cells (Kim et al., 1999). Similarly, in callus cultures of the legume plant *Ononis arvensis*, flavonoid levels increased after elicitation with $CuSO_4$, but also with $CdCl_2$ (Tumova and Ruskova, 1998).

While plants are in need of some transition metals such as copper or zinc, other metals such as lead or cadmium are highly toxic. Therefore, plants need to adjust their

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metal homeostasis accordingly to limit exposure to the more toxic compounds (Clemens, 2001). Plants have evolved sophisticated mechanisms to deal with toxic levels of metals in the soil. In hyperaccumulator plants such as *Thlaspi caerulescens* the uptake of heavy metals by specialized transporters also plays a role (Kochian et al., 2002). Also, other heavy metal binding proteins such as metallothioneins, small molecules which can effectively bind heavy metals (Cobbett, 2000), are included in the strategy to tolerate heavy metals. It was shown that in *Arabidopsis thaliana* a family of putative selenium-binding protein (SBP) genes was differentially upregulated in Cd-treated seedlings (Dutilleul et al., 2008). In plants and yeast the dominant pathway for metal detoxification is via phytochelatins (Grill et al., 1985; Ha et al., 1999; Clemens and Simm, 2003). Phytochelatins are small peptides synthesized non-translationally from glutathione catalyzed by phytochelatine synthase (reviewed in Clemens, 2001). The phytochelatin synthase mutant *cad1* of *Arabidopsis* is for example more sensitive to Cd than the wild type Howden et al., 1995). In *Arabidopsis* there are two genes *AtPCS1* and *AtPCS2* with non-redundant function because overexpression of *AtPCS2* in the *cad1* mutant could not rescue the phenotype (reviewed in Clemens, 2006). However, overexpression of *AtPCS1* in tobacco increased heavy metal tolerance (Wojas et al., 2008).

More is known about heavy metal tolerance in hyperaccumulators closely related to the model plant *Arabidopsis thaliana*, e.g. *Arabidopsis halleri* (Roosens et al., 2008). However, to study a situation in which tolerance could be enhanced by adding bioactive compounds a plant species with moderate tolerance is needed. In this work the possible relation between flavonoids and heavy metal tolerance was therefore investigated in *Arabidopsis thaliana* based on the information in the literature that flavonoids could: a) act as metal chelators and b) as antioxidants to protect the plant against oxidative stress caused by high levels of heavy metals.

MATERIALS AND METHODS

Plant Material

The ecotype Ler of *Arabidopsis thaliana* as well as the *transparent testa* (*tt*) mutants used in this study were provided by the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK). Seeds were surface sterilized with commercial bleach (active NaOCl in final solution 1%) for 20 min and then rinsed with sterile distilled water. Ten seeds were placed on each plate, and for each condition (see below) four plates were used.

Plant cultivation

Plants were cultivated on full strength MS-medium including vitamins (Murashige and Skoog, 1962) containing 1% sucrose and 1.2% phytoagar (Duchefa) and a final pH of 5.8 under long day conditions (16 h light / 8 h dark cycle), 24°C for 15 days. Heavy metals were administered as zinc chloride ($ZnCl_2$) and cadmium chloride ($CdCl_2$) as 0.1 M aqueous stock solution with final concentrations in the agar medium as given in results. The flavonoids naringenin and quercetin (Sigma) were dissolved in dimethylsulfoxide (DMSO) as 10 mM stock solutions and stored at 4°C. Stock solutions were diluted in MS medium to achieve final concentrations of 10^{-4} and 10^{-5} M. The flavonoids were administered to the plates one week after germination by pipetting the respective amount of the stock solution to the plates, which contained already the seedlings on heavy metal agar. The plates were dried open under a sterile bench, and after 45 min the liquid was dried down, and the plates were again closed with parafilm.

Growth analysis

Root growth was measured for each individual seedling (40 seedlings were measured for one treatment) at two time points: 1) at the time point when flavonoids were added (7 days after germination) and 2) 15 days after germination. For fresh weight determination, plants were carefully removed from the plates so that residues would not be left on the agar and then dried with filter paper. For fresh weight determination all seedlings from one plate were weighed as one sample (four plates were analyzed per treatment). Mean values were calculated. The significance of differences was determined using the Tukey test with a 5% confidence interval.

RNA isolation and RT-PCR

For RNA extraction seedlings were carefully removed from the plates, dried with filter paper and frozen in liquid N_2. Total RNA was obtained by using the TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized by reverse transcription polymerase chain reaction (RT-PCR). The primer sequences are perfect match primers corresponding to a non-homologous region of the other family member gene. To exclude the amplification of genomic DNA the RNA was digested with RNAse free DNAse (Promega). The following primers were used for amplification of *AtPCS1* (At5g44070): forward 5′-TCCGAAGCGATTCAGAAAGT-3′, reverse: 5′-TGATGAATCCGAGAAACCAG-3′, which resulted in an amplificate of 555 bp; for AtPCS2 (At1g03980): forward 5′-AGCCAGCTTTTTGTGGCTTA-3′, reverse: 5′-CGAATCTCAGCCATCCATTT-3′, which yielded a 721 bp fragment. To confirm equal amounts of cDNA the samples were amplified with *Arabidopsis thaliana Actin2* (At3g18780) primers. PCR was performed according to standard procedures using the following parameters for individual primer combinations: *AtPCS1* annealing temperature 55°C, elongation time 35 s, number of cycles 27; *AtPCS2* annealing temperature 58°C, elongation time 70 s, number of cycles 33; *AtActin2* annealing temperature 56°C, elongation time 60 s, number of cycles 24. The cycle number was adjusted so that analysis was performed during the exponential phase. PCR products were quanti-

fied after gel electrophoretic separation on 1.2% agarose gels and staining with ethidium bromide using Image J (http://rsb.info.nih.gov.ij). Mean values ± SE are given.

In vivo staining of flavonoids

Flavonoids were visualized *in vivo* by the fluorescence of flavonoid-conjugated DPBA (diphenylboric acid 2-amino-ethylester) to the compounds after excitation with blue light as decribed by Buer and Muday (2004). DPBA fluoresces orange (emission maxima [E_{max}] = 543 nm) when bound to quercetin and yellow-green (E_{max} = 520 nm) when bound to kaempferol. Whole seedlings were directly stained for 5 min using saturated (0.25%, w/v) DPBA and 0.005% (v/v) Triton X-100. Seedlings were then washed for 5 min with 100 mM sodium phosphate buffer, pH 7.0 containing 0.005% (v/v) Triton X-100. Fluorescence was visualized by excitation with DAPI filters (G365/ FT395/ LP420 nm) on a Zeiss Axioskop 2 (Carl Zeiss, Jena, Germany).

Antioxidative activity

Scavenging activity of flavonoids on 1,1'-diphenyl-2-picrylhydrazyl (DPPH) was measured according to the method reported previously (Blois, 1958). The scavenging activity of the test compound was measured as the decrease in DPPH absorbance at 517 nm and expressed as a percentage of the absorbance of a control DPPH solution without the test compound.

RESULTS AND DISCUSSION

Flavonoids have multiple protective functions such as antioxidative activity (Aherne and O'Brien, 2000; Havsteen, 2002; Rusak et al., 2005). Therefore, they have also been discussed as protectants against heavy metal stress (Tumova and Ruskova, 1998; Kim et al., 1999; Michalak, 2006). Phenolics, especially flavonoids, can be oxidized by peroxidase, and act in the H_2O_2-scavenging, phenolic/ASC/POX system upon heavy metal treatment (Michalak, 2006). Heavy metal toxicity can be a result of: 1) production of reactive oxygen species by autoxidation and Fenton reaction (typical for transition metals such as iron or copper), 2) blocking of essential functional groups in biomolecules (mainly non-redox-reactive heavy metals such as cadmium and mercury), and 3) displacement of essential metal ions from biomolecules, which occurs with different kinds of heavy metals (Schützendübel and Polle, 2002). Heavy metal stess can therefore increase the reactive molecules in the stressed tissue, which in turn should activate the antioxidative system of the cell. However, transition metals initiate hydroxyl radical production, which can not be controlled by antioxidants. Exposure of plants to non-redox reactive metals also results in oxidative stress as indicated by lipid peroxidation, H_2O_2 accumulation, and an oxidative burst. Cadmium and some other metals cause a transient depletion of GSH and an inhibition of antioxidative enzymes, especially of glutathione reductase (Schützendübel and Polle, 2002). Therefore, alternative antioxidants, such as flavonoids, may be produced (Tumova and Ruskova, 1998; Kim et al., 1999; Michalak, 2006). Flavonoids are known to form complexes with heavy metals, and this could lead to an effective addition to the plant's repertoire of defensive responses to heavy metal stress (Brown et al., 1998; Aherne and O'Brien, 2000; Soczynska-Kordala et al., 2001; Michalak, 2006; Korkina, 2007). Therefore, the involvement of flavonoids in heavy metal tolerance was inves-

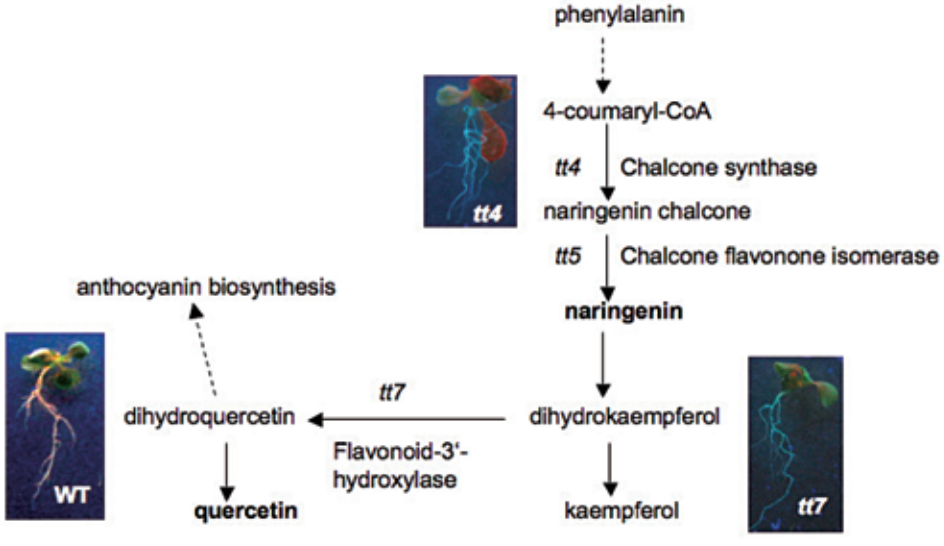

Figure 1. The biosynthetic pathways leading to the flavonoids naringenin and quercetin (in bold) used in this study. The position of the mutated genes of the *transparent testa* mutant lines in the pathway is indicated. Staining of flavonoids with DPBA revealed orange fluorescence typical for quercetin in wild type (WT) and green fluorescence typical for kaempferol in *tt7* roots. *tt4* (and *tt5*, not shown) mutants show light blue fluorescence typical for sinapate derivatives. Red fluorescence is from chlorophyll.

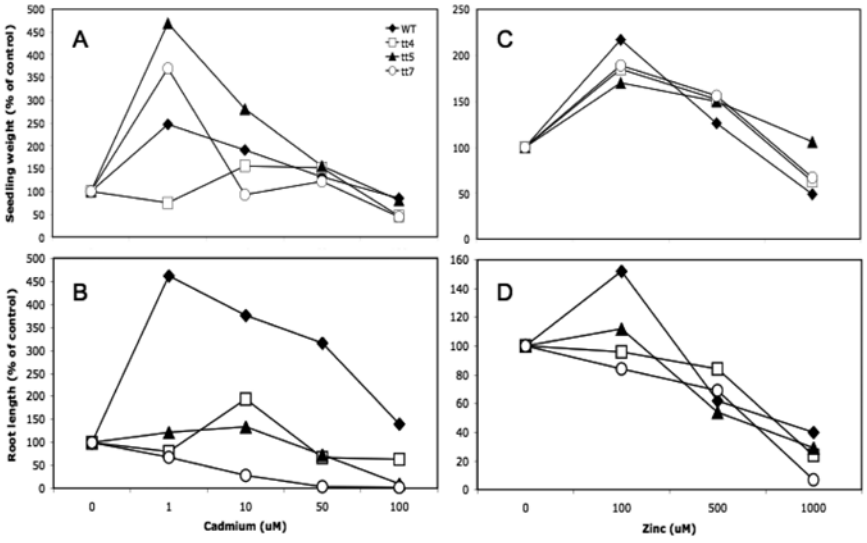

Figure 2. Growth of wild type and flavonoid deficient mutant lines (*tt4*, *tt5*, *tt7*; for the defect in the respective enzymatic reaction see Figure 1) on different concentrations of cadmium (A, B) and zinc (C, D). In A and C the weight of the total seedling is shown as percent of control, in B and D the root length as percent of control is depicted. (◆) WT, (□) *tt4*, (▲) *tt5*, (○) *tt7*.

tigated using the model plant *Arabidopsis thaliana*. The main flavonoids in *Arabidopsis* are the flavanone naringenin and the flavonols quercetin and kaempferol and their respective glycosides (Winkel-Shirley, 2001) (Figure 1). While naringenin did not show antioxidative activity with the radical DPPH, quercetin was highly active in scavenging radicals (data not shown). These two flavonoids were therefore used to distinguish between general antioxidative activity and other mechanisms to influence heavy metal tolerance in *Arabidopsis*.

Wild type (WT) and flavonoid deficient mutants (*tt4*, *tt5*, *tt7*) were investigated with respect to their heavy metal tolerance on either cadmium (Cd) or zinc (Zn) at different concentrations. The mutants *tt4* and *tt5* do not possess any flavonoids due to their defects in chalcone synthase and chalcone isomerase, respectively (Figure 1). The line *tt7* still makes naringenin and kaempferol, but no quercetin because of the defect in flavonoid-3'-hydroxylase. If flavonoids are important factors in heavy metal tolerance in *Arabidopsis*, then the mutant lines should be more sensitive to heavy metal treatment than the WT. To test this hypothesis, WT and flavonoid deficient mutant lines were grown on different concentrations of Cd and Zn (1-100 μM and 100-1000 μM, respectively). While Cd was generally already growth inhibitory at low concentrations, for Zn higher concentrations had to be administered (Figure 2). Two parameters were evaluated: root length and seedling fresh weight. The seedling weight and root length was considerably inhibited in *tt* mutant lines on Cd while the WT was even stimulated in root growth at low concentrations (Figure 2A, B). A similar effect was observed on low Cd with tomato plants, where low Cd increased the length of shoots, but decreased growth at higher concentrations (Dong et al., 2005). It is interesting to note that the root length of *tt7*, the mutant line with naringenin and kaempferol present, showed the least inhibition on Cd of all mutant lines, the values lying between WT and the two flavonoid deficient mutant lines *tt4* an *tt5* (Figure 2A). On

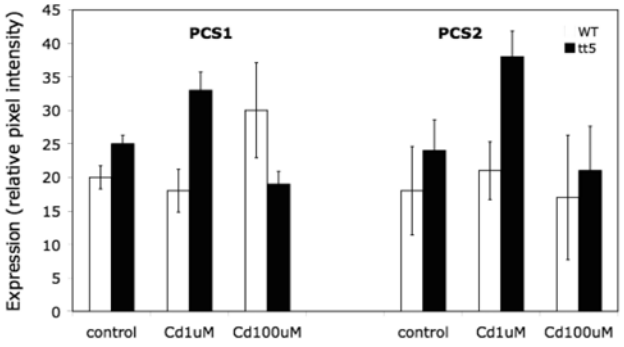

Figure 3. Expression analysis of two phytochelatin synthase (PCS) genes in *Arabidopsis* wild type seedlings and the flavonoid deficient mutant *tt5* under control conditions and after growth on two different Cd concentrations. The expression of *AtPCS1* and *AtPCS2* was determined with semi-quantitative RT-PCR and the values normalized on *AtActin2* as housekeeping gene (data not shown). Mean values ± SE of three independent measurement are given. (□) WT, (■) *tt5*.

Zn root growth was also lower in mutants than in the WT at 100 μM Zn (Figure 2D) while seedling weight was the same for all lines (Figure 2C).

As a marker for heavy metal tolerance the expression levels of the two phytochelatin synthase genes *PCS1* and *PCS2* were analyzed after treatment with Cd in WT and *tt5* mutant seedlings (Figure 3). In WT only *PCS1* was induced at high Cd concentrations (100 μM). *PCS2* was not induced, although the expression levels of *PCS1* and *PCS2* under control conditions were similar. Since *PCS1* overexpression was already sufficient to introduce heavy metal tolerance (Howden et al., 1995; Clemens, 2001, 2006), expression of *PCS2* is probably not necessary. In contrast, transcripts were induced in both the flavonoid deficient *tt5* mutant *PCS1* and *PCS2*. The induction could be found already at 1 μM (Figure 3). It is tempting to speculate that upregulation of both PCS genes is an effort to partially

compensate for the missing flavonoids. However, flavonoids themselves are not involved in the induction of *PCS* genes because the upregulation occurs in *tt5* plants as well. Therefore, alternative systems for heavy metal detoxification or export (Kochian et al., 2002; Dutilleul et al., 2008) should be investigated in these mutants.

It was then examined whether flavonoids are inducible by heavy metal treatment using an *in vivo* staining method (data not shown). While WT and *tt7* seedlings showed the expected fluorescence stain of quercetin (kaempferol was not visible in WT due to the strong fluorescence of quercetin) and kaempferol, respectively, *tt4* (Figure 1) and *tt5* (data not shown) showed only the light blue fluorescence of sinapate derivatives. No further increase in fluorescence was observed when the WT and *tt7* plants were grown on heavy metal containing agar (data not shown). Since other authors found an induction of flavonoids upon heavy metal treatment (Tumova and Ruskova, 1998; Kim et al., 1999; Michalak, 2006), further investigations should also take measurement of endogenous flavonoids into account because quantitative differences can not be visualized by the in vivo staining method.

Finally, it was investigated whether addition of the flavonoids naringenin and quercetin could rescue the growth inhibition in either WT or mutant seedlings. The influence of flavonoids was tested at 10 and 100 μM in addition to

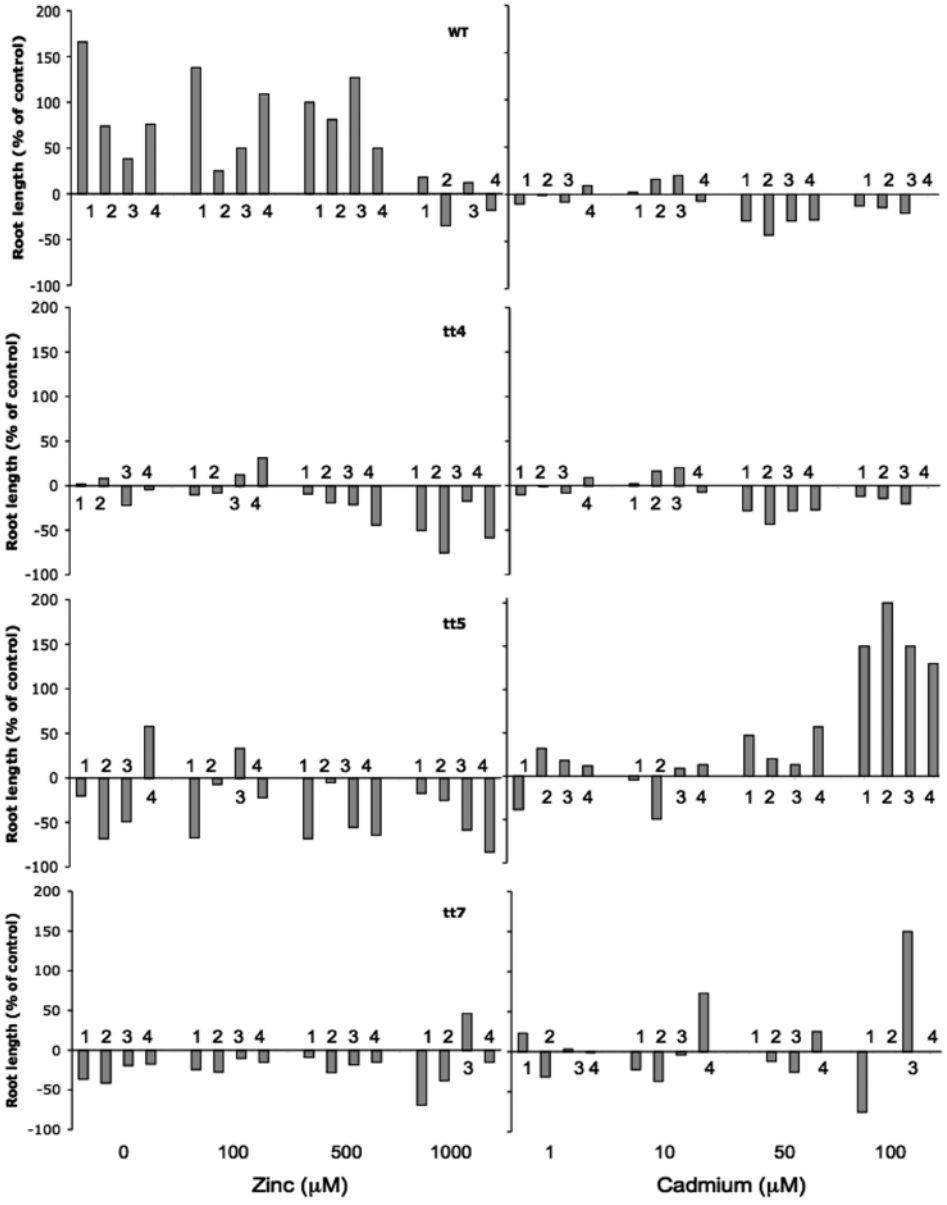

Figure 4. Effects of the two flavonoids naringenin and quercetin on root length of *Arabidopsis* wild type and mutant seedlings (*tt4*, *tt5*, *tt7*) under the influence of different zinc or cadmium concentrations. All values are given as percentage of the respective control treatment. (1) 10 μM naringenin, (2) 100 μM naringenin, (3) 10 μM quercetin; (4) 100 μM quercetin. Values above zero mean induction above level of treatment with heavy metal only, negative values (below zero) indicate inhibition.

heavy metal treatment. To show the differences for the individual treatments better, the effect of narigenin and quercetin was given as percentage of the respective control (without or with heavy metal treatment). Root growth of WT seedlings was close to the control treatment on 100 and 500 μM Zn (Figure 4). None of the mutant lines could be stimulated by the addition of either flavonoid. However, *tt5* seedlings showed increased root growth under high Cd concentrations when simultaneously incubated with both flavonoids, which also happened for *tt7* with high quercetin concentrations (Figure 4). When total seedling weight was used as a parameter to evaluate heavy metal tolerance, all flavonoids could partially rescue the growth inhibition phenotype, especially in line *tt5* and, under high Zn concentrations, also in *tt4* (Figure 5). The addition of flavonoids had only a moderately positive influence on the growth of Line *tt7*. On Cd the effect of flavonoids was again most prominent for line *tt5*, followed by WT, and to a lesser extent by lines *tt4* and *tt7*. Overall, no prominent differences between naringenin and quercetin emerged when all lines were monitored. Therefore, it can be assumed that the effect is not due to the antioxidative potential present with quercetin but not naringenin. However, the protective potential found for both naringenin and quercetin could be also due to other properties of these molecules.

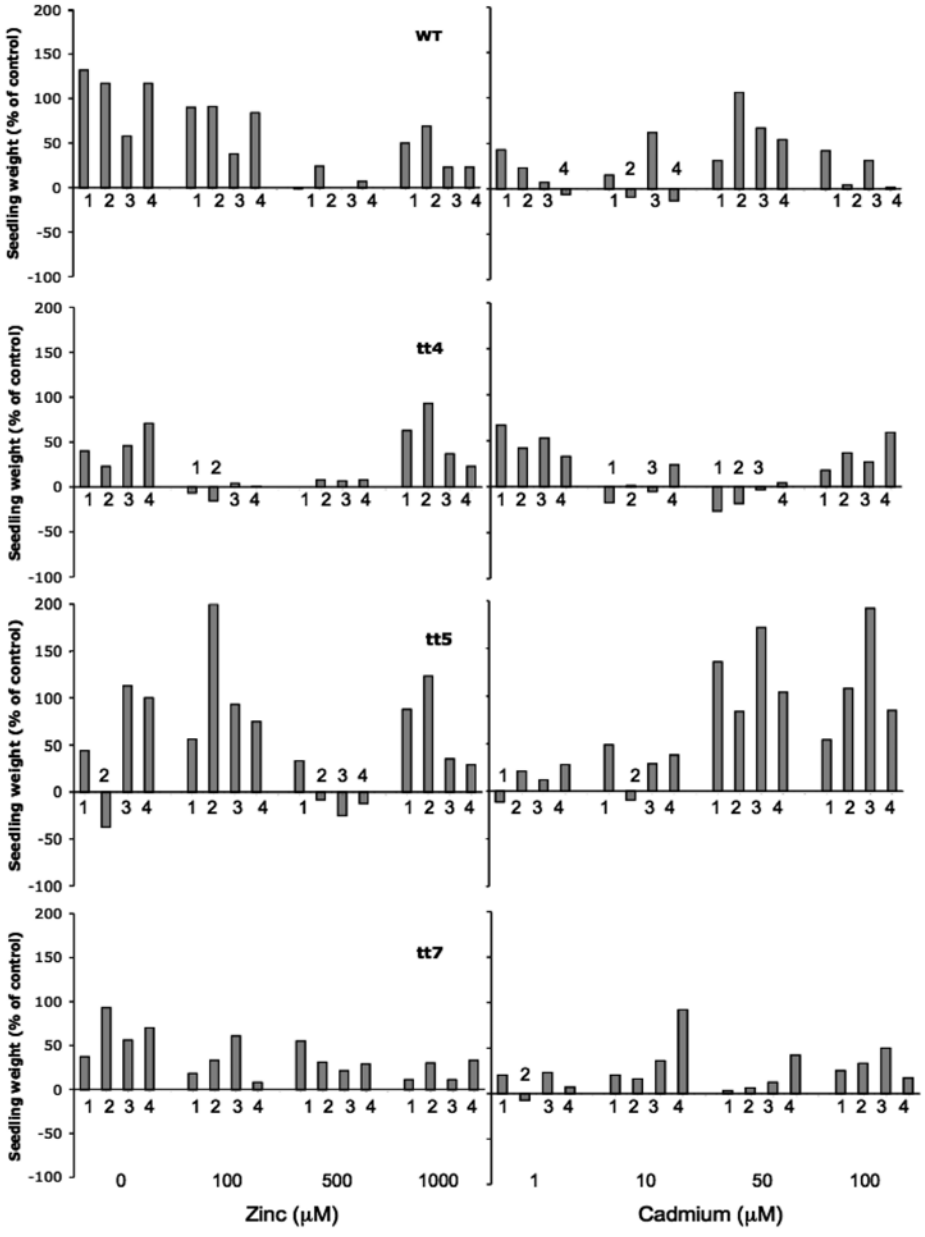

Figure 5. Effects of the two flavonoids naringenin and quercetin on seedling fresh weight of *Arabidopsis* wild type and mutant seedlings (*tt4*, *tt5*, *tt7*) under the influence of different zinc or cadmium concentrations. All values are given as percentage of the respective control treatment. (1) 10 μM naringenin, (2) 100 μM naringenin, (3) 10 μM quercetin; (4) 100 μM quercetin. Values above zero mean induction above level of treatment with heavy metal only, negative values (below zero) indicate inhibition.

While exogenous application of flavonoids might not be sufficient for protecting against heavy metal stress because of possible problems with uptake and distribution of flavonoids to the appropriate tissues, e.g. in the mutant lines, overexpression of genes coding for components of flavonoid biosynthesis or transcription factors (e.g. Stracke et al., 2007) could be a fruitful strategy for elucidating their role in heavy metal tolerance.

In conclusion, our study has provided evidence that flavonoids could be an additional factor in heavy metal tolerance in *Arabidopsis thaliana*. Naringenin and quercetin were able to restore partial seedling growth in wild type plants, but also in *tt5* mutants. In the other two flavonoid-deficient mutants, this was not the case.

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類黃素對阿拉伯芥幼苗之重金屬耐受性之效果

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本文使用阿拉伯芥為材料來檢驗類黃素對它的重金屬耐受性之可能影響。野生型 WT 及突變種 (*tt*, 類黃素之生合成受損者) 分別栽培於含不同重金屬之培養基並且評估兩種生長參數。當 Cd 存在時，*tt* 之根長及幼苗重都比 WT 減少 ； 當 Zn 存在時只有根長減少。做為重金屬耐受性之標誌，我們以 RT-PCR 定量兩種 phytochelatin synthase 基因 (*AtPCS1*, *AtPCS2*) 之誘導。在較高濃度之 Cd 存在下，*AtPCS1* 之轉錄產物在 WT 高於 *tt5*；相反地，*AtPCS2* 之轉錄產物並未在 WT 產生，但卻在 *tt5* 產生。接著我們檢查突變種之生長受抑制之表現型可否以添加類黃素之方式來補救。為此，幼苗分別以 flavanone naringenin 及 flavonol quercetin 處理。對 WT 而言，quercetin 及 naringenin 在 Zn 濃度高達 500 μM 時仍可促進根之生長及幼苗之鮮重，然而對比而言，卻無效果。可是幼苗重有所改善，尤其是 *tt5*。當 Cd 存在時，只有 tt5 之根長缺陷可被改善，而幼苗重在所有品系之植物都有改善，只是程度不同而已。上述結果以 flavonoid 在重金屬耐受性方面之潛在功能予以討論。

關鍵詞 ： 抗氧化劑 ； 阿拉伯芥 ； 重金屬耐受性 ； 類黃素 ；phytochelatin 合成酶 ； 透明種膜之突變種。