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Leaf nutrient homeostasis and maintenance of photosynthesis integrity contribute to adaptation of the pea mutant SGECd^t to cadmium

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Abstract

Cadmium (Cd) is a highly toxic and widespread soil pollutant, which negatively affects various aspects of plant growth and physiology. Here, the role of photosynthesis in response to Cd was investigated in the Cd-tolerant pea (*Pisum sativum* L.) mutant SGECd^t. The wild type SGE and the mutant SGECd^t were grown in a hydroponic solution supplemented with 1, 3, or 4 μM $CdCl_2$ for 12 d. Root and shoot biomasses of the Cd-treated SGECd^t were significantly higher than of SGE. Cadmium had little effect on the quantum yield of photosystem II (ϕPSII) and chlorophyll content of intact leaves of both pea genotypes. However, when leaf slices were taken from Cd-exposed plants and incubated with high Cd concentrations, the SGECd^t mutant showed 1.5 - 2 times higher ϕPSII values than SGE, with genotypic differences maximal at 0.1 and 1 mM $CdCl_2$. In contrast, when leaf slices were taken from plants previously unexposed to Cd, both pea genotypes exhibited similar ϕPSII values. Cadmium content in leaves and mesophyll protoplasts of Cd-treated SGECd^t were about 2 - 3 times higher than in SGE ones. The mutant leaves and mesophyll protoplasts had also higher Ca, Mg, Mn, and Zn content. Thus, SGECd^t acclimated to Cd during growth in the Cd-supplemented nutrient solution by developing a molecular mechanism related to photosynthetic integrity. A higher foliar nutrient content likely allows enhanced photosynthesis by counteracting the damage of leaves caused by Cd.

Additional key words: calcium, chlorophyll, magnesium, manganese, protoplast, quantum yield of photosystem II, zinc.

Introduction

Cadmium (Cd) is one of the most toxic and widely spread soil pollutants. Cd affects plant growth and metabolism and inhibits photosynthesis in various plants (Krupa *et al.* 1993, Böddi *et al.* 1995, Ouzounidou *et al.* 1997, Haag-Kerwer *et al.* 1999, Baryla *et al.* 2001, Pietrini *et al.* 2003, Drazkiewicz *et al.* 2003, Kevrešan *et al.* 2004, Zhou and Qui 2005) including pea (Sandalio *et al.* 2001, Balakhnina *et al.* 2005). Cd-induced inhibition of photosynthesis is apparent from various photosynthesis-related processes, in particular lowered chlorophyll *a* fluorescence (*e.g.* in pea, see Balakhnina *et al.* 2005), inhibited rates of photosynthetic electron transport (Küpper *et al.*2007) and Calvin cycle reactions (Krupa *et al.* 1993), decreased chlorophyll biosynthesis (Böddi *et al.* 1995), impaired integration of chlorophyll into proteins (Horvath *et al.* 1996), inhibition of photosynthetic enzymes (Krupa *et al.* 1995, Siedlecka *et al.* 1998) and substitution of Mg^{2+} in chlorophyll (Shaul 2002).Thus, various photosynthesis-related markers can indicate Cd toxicity in plants.

Plants have evolved a number of mechanisms to tolerate and/or avoid toxic effects of Cd (Sanita di Toppi and Gabrielli 1999, Hasan *et al.* 2009, Lin and Aarts 2012). Genetics offers powerful tools to study mechanisms protecting plants against Cd and other toxic heavy metals. Several mutants with increased heavy metal sensitivity have been described. The Cd-sensitive mutants *cad1* and *cad2* of *Arabidopsis thaliana* are deficient in phytochelatin synthase (Howden *et al.* 1995, Ha *et al.* 1999) and γ-glutamylcysteine synthetase (Cobbet *et al.* 1998), respectively, demonstrating the important role of phytochelatins in Cd detoxification. Increased Cd uptake rates of the Cd-sensitive rice mutant significantly inhibited growth (He *et al.* 2007). Important roles of the ascorbate-glutathione cycle, antioxidant enzymes, and phytochelatins in tolerance and accumulation of Cd by plants were revealed in the Cd-tolerant rice mutant

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Abbreviations: ϕPSII - quantum yield efficiency of photosystem II; SGE - wild type pea line; SGECd^t - Cd-tolerant pea mutant.
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cadH-5 (Shen *et al.* 2012). The increased Cd and Pb tolerance of the *A. thaliana* mutant *cdr3-1D* was explained by low metal accumulation due to active expression of *AtPDR8*/*AtPDR12* genes, encoding transporters responsible for excluding these metals from the cytoplasm, as well as by activating glutathione biosynthesis (Wang *et al.* 2011). Three *A. thaliana* mutants showed distinctly different Cd responses: the mutant *MRC-32* exhibited enhanced tolerance to and accumulation of Cd, *MRC-22* showed a Cd-phobic response of roots, and *MRC-26* accumulated less Cd in the shoot (Watanabe *et al.* 2010). However, the mechanisms causing these phenotypic changes need to be elucidated. Enhanced copper accumulation in plant tissues caused greater Cu sensitivity of the *A. thaliana* mutant *cup1* (Van Vliet *et al.* 1995). The pea mutant E107 (*brz*) had greater Fe (Welch and LaRue 1990) and Pb (Chen and Huang 2007) accumulation and decreased Al tolerance (Guinel and LaRue 1993) due to acidification of the rhizosphere. Active formation of root cell wall material binding Pb caused increased Pb accumulation in the *Brassica juncea* mutant *7/15-1* (Schulman *et al.* 1999).

The pea mutant SGECd^t carries a recessive mutation in one gene and shows increased Cd tolerance and Cd accumulation (Tsyganov *et al.* 2007). Although the mutated gene in SGECd^t is still not found, the proposed mechanisms involved in its Cd tolerance were related to maintenance of biochemical and nutrient homeostasis (Tsyganov *et al.* 2007, Belimov *et al.* 2016), water relations (Belimov *et al.* 2015) and proper root growth and function (Belimov *et al.* 2018) under Cd stress. Therefore, an important next step was to study the effects of Cd on plant photosynthesis using this pea mutant and to determine its photosynthetic activity in order to better understand the mechanisms involved in plant response to toxic heavy metals. Whether the enhanced Cd accumulation of this mutant induced cellular tolerance mechanisms was investigated by comparing the photosynthetic responses to Cd in tissues from control (previously not exposed to Cd) and Cd-treated plants *in vitro*.

Materials and methods

Plants and cultivation: The laboratory pea (*Pisum sativum* L.) line SGE and its Cd-tolerant mutant SGECd^t (Tsyganov *et al.* 2007) originated from the collection of the All-Russia Research Institute for Agricultural Microbiology were propagated by the authors.

Seeds were surface-sterilised and scarified by treatment with 98 % (m/v) H_2SO_4 for 30 min, rinsed carefully with tap water, and germinated on filter paper at 25 °C in the dark for 3 d. Seedlings were transferred to plastic pots (2 pots with 3 seeds per genotype and treatment) containing 500 cm^3 of nutrient solution [μM]: KH_2PO_4, 110; $Ca(NO_3)_2$, 50; $MgSO_4$, 400; KCl, 300; $CaCl_2$, 70; NaCl, 5; Fe-tartrate, 2; H_3BO_3, 1; $MnSO_4$, 1; $ZnSO_4$, 1; Na_2MoO_4, 0.03; $CuSO_4$, 0.8; pH 5.5. $CdCl_2$ was added to the solution the next day in final concentrations of 0, 1, 3, or 4 μM. Experiments with 0 and 3 μM $CdCl_2$ were performed at Bielefeld University (Bielefeld, Germany) and experiments with 0, 1 and 4 μM $CdCl_2$ performed at Lancaster University (Lancaster, UK). The plants were cultivated for 12 d in a growth chamber at a 12-h photoperiod, a photon flux density of 200 μmol (photons) m^{-2} s^{-1}, day/night temperatures of 23/18 °C, and a relative humidity between 50 and 60 %. The non-aerated nutrient solution was replaced twice, after 5 and 8 d of Cd exposure. Protoplasts were isolated from leaves of plants treated with 3 μM $CdCl_2$. Chlorophyll content of second true leaves were determined as described by Dietz *et al.* (1992). The root and shoot fresh mass (FM) and dry mass (DM) were determined for each plant.

Quantum yield efficiency of photosystem (PS) II: Second true leaves were sliced into pieces (about 1 × 6 mm) with a sharp razor, and floated on solutions of increasing Cd-concentrations in the dark (0, 0.1, 1, and 10 mM $CdCl_2$). Chlorophyll *a* fluorescence emission was used to calculate the quantum yield efficiency of PS II (ϕPSII) after 6-h Cd-treatment (Cho *et al.* 2003). Three experiments were performed with 18 determinations for each genotype and treatment using the pulse amplitude-modulated chlorophyll fluorescence meter *PAM 100* (*Heinz Walz*, Effeltrich, Germany). The same method was used for ϕPSII determination of intact leaves the day before harvest in two experiments with about 100 determinations for each genotype and treatment using the plant efficiency analyser (*Hansatech Instruments*, King's Lynn, Norfolk, UK).

Protoplast isolation and analysis: Protoplasts were isolated as described by Tsyganov *et al.* (2007). Briefly, leaves were sliced into 2-mm sections with a sharp razor and vacuum-infiltrated with 10 cm^3 of sorbitol medium with 400 mM sorbitol, 20 mM KCl, 10 mM $CaCl_2$ and 20 mM MES (pH 5.7) supplemented with 1.5 % (v/v) cellulase *Onozuka R10* and 0.4 % (v/v) *Macerozyme R10*, and incubated in Petri dishes at 30 °C in the dark for 2.5 h. The released protoplasts were filtered and layered on top of 1 cm^3 of *Percoll*® medium (400 mM sorbitol, 20 mM KCl, 10 mM $CaCl_2$, 20 mM MES, pH 5.7). A discontinuous gradient was formed by successively layering 40 %, 30 %, and 20 % *Percoll* medium and sorbitol medium on top. After centrifugation at 4 °C for 3 min each at 800 *g* and 3000 *g*, the protoplast fraction was collected from the interphase at 40 % and 30 % *Percoll*. Chlorophyll content in protoplast suspensions was determined as by Dietz *et al.* (1992).

Plant elemental analysis: The protoplasts were digested in 10 % HNO_3 (v/v) at 165 °C under pressure. The extracts were analysed for Cd and nutrients content with an inductively coupled plasma atomic emission spectrometer (*Jobin Yvon JY 70*, *Instruments S.A.*, Longjumeau, France). Leaves were dried, ground and digested in a mixture of concentrated HNO_3 and 38 % H_2O_2 at 70 °C using *DigiBlock* (*LabTech*, Sorisole, Italy). Concentrations of elements in digested leaves were determined using an inductively coupled plasma emission spectrometer *ICPE-9000* (*Shimadzu*, Kyoto, Japan).

Statistical analysis and data calculations: The data were processed by analysis of variance (*MANOVA*) using

the software *STATISTICA v. 10.0* (*TIBCO Software Inc.*, Palo Alto, CA, USA). Standard errors (SEs) and standard deviations (SDs) were calculated. The Fisher's least significant difference test (one way *ANOVA*) was used to evaluate differences between means.

The estimated distribution of elements in the mesophyll protoplasts relative to leaves was calculated as a relative share of element (RSE) using a formula: $RSE = [(E_{MP} \times Cd_L) / (E_L \times Cd_{MP})] \times PL_{Cd}$, where E_{MP} is element content in mesophyll protoplasts, E_L is element content in leaves, Cd_{MP} is Cd content in mesophyll protoplasts, Cd_L is- Cd content in leaves, PL_{Cd} is assumed percent localization of Cd in mesophyll protoplasts. Two scenarios were calculated based on the assumption of 100 % PL_{Cd} or 75 % PL_{Cd}. Cadmium was chosen in this formula because it was the element with the highest relative abundance in the mesophyll protoplasts. By this way, the estimated distribution (relative share) of elements in the mesophyll protoplasts was obtained in the percentage of total.

Results and discussion

Treatment with 3 μM Cd significantly reduced root length and biomass of both pea genotypes (Table 1). Cd-treated SGECd^t mutant had 1.8-fold longer roots and 2.4-fold more biomass than SGE. Cd also inhibited shoot growth of SGE by 35 %, whereas shoot biomass of SGECd^t was not affected. These data agree with our previous results where plants were cultivated at 3 μM Cd (Tsyganov *et al.* 2007). In the second set of experiments, two Cd concentrations were chosen resulting in similar growth inhibition of SGE at 1 μM Cd and SGECd^t at 4 μM Cd, in aiming to induce the same physiological reactions in both genotypes (Table 2).

When the plants were grown at 3 μM Cd, the ϕPSII of intact leaves (measured in the afternoon) was slightly decreased only in the mutant by 4 % and no genotypic differences were observed in the presence or absence of Cd (Table 1). Treatment with 1 μM Cd slightly decreased ϕPSII of intact SGE leaves measured in the morning and afternoon by 3 % (Table 3). SGECd^t treated with 1 μM Cd showed slightly increased ϕPSII by 3 % as compared to SGE treated with 4 μM Cd. Although the described differences between treatments or genotypes were statistically significant, they did not exceed 5 %.

Using chlorophyll *a* fluorescence emission to study Cd effects on photosynthesis gives variable results depending on Cd concentration, exposure time, and plant species. A short (2 h) exposure of pea roots to high Cd concentration (1 mM) halved the variable fluorescence (F_v) in leaves (Balakhnina *et al.* 2005). However, a long exposure (about 10 d) to relatively low Cd concentrations (up to 50 μM depending on plant species) had no effect on chlorophyll *a* fluorescence in leaves of various species, such as *Phaseolus vulgaris* (Krupa *et al.* 1993), *Triticum aestivum* (Ouzounidou *et al.* 1997), *Brassica juncea* (Haag-Kerwer *et al.* 1999), *Phragmites australis* (Pietrini *et al.* 2003), *Zea mays* (Drazkiewicz *et al.* 2003), and the Cd-hyperaccumulating plant *Sedum alfredii* (Zhou and Qui 2005). Overall the effects of Cd on chlorophyll *a* fluorescence were small.

A detailed study of chlorophyll fluorescence parameters using the hyperaccumulating plant *Thlaspi caerulescens* showed that Cd inhibited the photosynthetic light reactions more than the Calvin cycle (Kupper *et al.* 2007). Chlorophyll *a* fluorescence of the legume crop *P. vulgaris* measured in dark-acclimated leaves was not affected by Cd, whereas in constant irradiance with open photosystem II reaction centres it was inhibited (Krupa *et al.* 1993). Therefore, the authors concluded that the Calvin cycle reactions were more likely than photosystem II to be the primary targets of Cd toxicity. However, in our experiments with pea the effect of Cd on intact leaves was independent of time of day for both genotypes. Therefore, the activity of photosynthesis in intact leaves was approximately the same in both genotypes in the presence or absence of Cd.

Chlorophyll content did not differ between genotypes for untreated plants and for plants treated with 1 μM Cd. However, 1 μM Cd decreased chlorophyll *a*+*b* content by 11 % and 4 μM Cd decreased chlorophyll *b* content by 8 % (Table 3). However, 4 μM Cd decreased chlorophyll *b* content more in the SGECd^t mutant (by 13 %) than in SGE, perhaps related to the faster shoot growth of SGECd^t in the presence of toxic Cd, since its shoot biomass was twice that of SGE (Table 2).

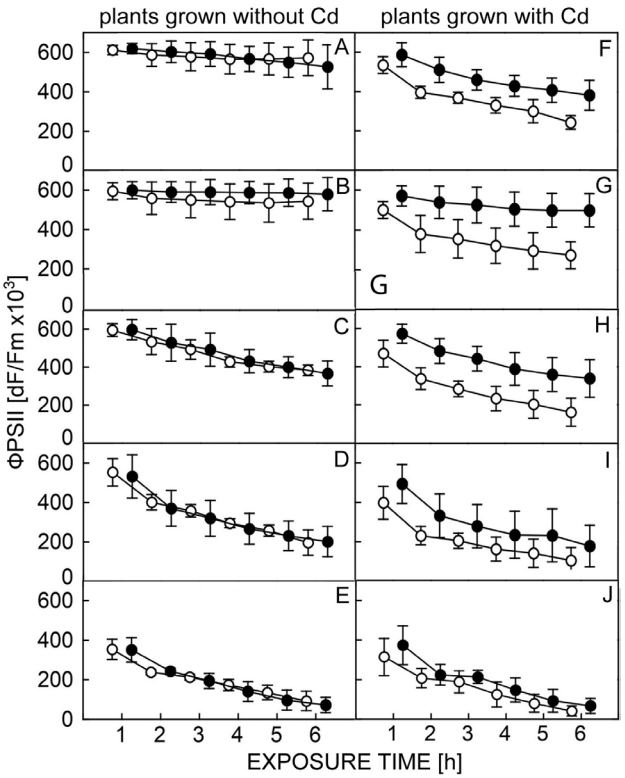

Fig. 1. Cadmium effect on quantum yield efficiency of photosystem II (ϕPSII) in leaf slices of wild type (SGE, ○) and mutant (SGECd^t, ●) pea. The plants were grown in a nutrient solution in the absence or presence of 3 μM $CdCl_2$ for 10 d. The leaf slices were treated with 0, 0.1, 1, or 10 mM $CdCl_2$ for 6 h. Data are means ± SDs of three experiments with six determinations each.

Table 1. Effect of cadmium (3 μM $CdCl_2$) on plant growth parameters and quantum yield efficiency of photosystem II (ϕPSII) in intact leaves of wild type (SGE) and mutant (SGECd^t) pea. Data are means ± SEs of three experiments; for growth parameters $n = 36$, for ϕPSII $n = 75$. Measurements were performed in the afternoon (3 - 4 pm). Different *letters* show significant differences between genotypes and treatments (Fisher's least significant difference test, $P \leq 0.05$).

Treatment	Genotype	Root length [cm]	Biomass [mg (plant f.m.)$^{-1}$]		ϕPSII [dF/Fm × 10^3]
			root	shoot	
Untreated plants	SGE	21 ± 0.6 ^{cb}	480 ± 21 ^{c}	581 ± 15 ^{b}	734 ± 6 ^{ab}
	SGECd^t	22 ± 0.6 ^{c}	450 ± 18 ^{c}	569 ± 16 ^{b}	749 ± 5 ^{b}
Treated with 3 μM $CdCl_2$	SGE	11 ± 0.4 ^{a}	109 ± 7 ^{a}	379 ± 13 ^{a}	717 ± 7 ^{a}
	SGECd^t	20 ± 0.6 ^{b}	265 ± 14 ^{b}	540 ± 19 ^{b}	717 ± 6 ^{a}

Table 2. Effect of cadmium (1 and 4 μM $CdCl_2$) on growth of wild type (SGE) and mutant (SGECd^t) pea. Data are means ± SEs of two experiments. Different *letters* show significant differences between genotypes and treatments (Fisher's least significant difference test, $P \leq 0.05$, $n = 20$).

Treatment	Genotype	Root length [cm]	Biomass [mg (plant d.m.)$^{-1}$]	
			Root	shoot
Untreated plants	SGE	15 ± 0.4 ^{b}	63 ± 2 ^{d}	175 ± 6 ^{d}
	SGECd^t	14 ± 0.4 ^{b}	60 ± 2 ^{d}	172 ± 5 ^{d}
Treated with 1 μM Cd Cl_2	SGE	14 ± 0.5 ^{b}	42 ± 2 ^{b}	145 ± 7 ^{c}
	SGECd^t	14 ± 0.3 ^{b}	54 ± 1 ^{c}	157 ±4 ^{c}
Treated with 4 μM $CdCl_2$	SGE	5 ± 0.1 ^{a}	11 ± 1 ^{a}	72 ± 2 ^{a}
	SGECd^t	14 ± 0.4^{b}	46 ± 1 ^{b}	138 ± 3 ^{c}

Table 3. Effect of cadmium (1 and 4 μM $CdCl_2$) on leaf chlorophyll content and photosynthesis in intact leaves of wild type (SGE) and mutant (SGECd^t) pea. Data are means ± SEs of two experiments. For chlorophyll content $n = 9$. ϕPSII - quantum yield efficiency of PS II in intact leaves ($n = 25$); it was measured in the morning (9 - 10 am) and in the afternoon (3 - 4 pm). Different *letters* show significant differences between genotypes and treatments (Fisher's least significant difference test, $P \leq 0.05$).

Treatment	Genotype	Chlorophyll content [μg mg^{-1}(leaf f.m.]			ϕPSII [dF/Fm × 10^3]	
		chlorophyll *a*	chlorophyll *b*	chlorophyll *a*+*b*	morning	afternoon
Untreated plants	SGE	0.71 ± 0.03 ^{b}	0.77 ± 0.03 ^{b}	1.48 ± 0.06 ^{b}	857 ± 3 ^{c}	822 ± 6 ^{b}
	SGECd^t	0.68 ± 0.02 ^{ab}	0.73 ± 0.02 ^{ab}	1.41 ± 0.03 ^{b}	856 ± 3 ^{c}	821 ± 7 ^{b}
Treated with 1 μM $CdCl_2$	SGE	0.68 ± 0.02 ^{ab}	0.71 ± 0.02 ^{ab}	1.39 ± 0.03 ^{ab}	829 ± 3 ^{a}	797 ± 8 ^{a}
	SGECd^t	0.62 ± 0.03 ^{a}	0.64 ± 0.02 ^{a}	1.26 ± 0.05 ^{a}	859 ± 2 ^{c}	824 ± 5 ^{b}
Treated with 4 μM $CdCl_2$	SGE	0.62 ± 0.02 ^{a}	0.76 ± 0.03 ^{b}	1.38 ±0.04 ^{ab}	854 ± 3 ^{bc}	807 ± 4 ^{ab}
	SGECd^t	0.63 ± 0.03 ^{ab}	0.66 ± 0.03 ^{a}	1.30 ± 0.06 ^{a}	848 ± 3 ^{b}	807 ± 8 ^{ab}

It was previously shown that Cd caused dramatic shoot ABA accumulation only in SGE thereby causing partial stomatal closure (Belimov *et al.* 2015). The lack of ABA accumulation in Cd-treated SGECd^t likely maintained photosynthesis, allowing continued shoot biomass accumulation. Total chlorophyll accumulation in mutant leaves at 4 μM Cd was also about twice that of SGE leaves (calculated as mg per plant using the data for shoot biomass (Table 2) and chlorophyll content given in Table 3). Although Cd decreased content of chlorophyll in leaves of various plant species (Krupa *et al.* 1993, Böddi *et al.* 1995, Baryla *et al.* 2001, Kevrešan *et al.* 2004), others found no effect of this metal on chlorophyll content in leaves of *Sedum alfredii* treated with up to 1 000 μM Cd for 20 d (Zhou and Qui 2005). Cadmium can decrease chlorophyll biosynthesis (Böddi *et al.* 1995) and/or disturb the integration of chlorophyll into stable chlorophyll-protein complexes (Horvath *et al.* 1996). In our experiments, Cd scarcely affected leaf chlorophyll content of both pea genotypes, suggesting that this parameter was not involved in Cd tolerance of SGECd^t.

Leaf slices of both pea genotypes grown without Cd exhibited similar responses of ϕPSII to treatment with different Cd concentrations during an experiment of 6 h duration (Fig. 1). The ϕPSII of the leaf slices treated with water or 0.1 mM $CdCl_2$ was unaffected in both genotypes. Treatment with 1 or 10 mM $CdCl_2$ decreased ϕPSII. For plants grown in the presence of Cd, ϕPSII of SGE leaf slices was 1.5 to 2-times lower and decreased more rapidly during Cd exposure (Fig. 1). SGE and SGECd^t leaf slices

Table 4. Cadmium and nutrient content of leaves and mesophyll protoplasts of Cd-treated wild type (SGE) and mutant (SGECd^t) pea. The plants were grown in nutrient solution in the presence of 1, 3 or 4 μM $CdCl_2$ for 12 days. Data are means ± SEs of one experiment with four determinations for leaves and two determinations for protoplasts. Different *letters* show significant differences between treatments within each treatment (Fisher's east significant difference test, $P \leq 0.05$). *Asterisks* show significant differences between plants treated with 1 and 4 μM $CdCl_2$ for the same pea genotype (Fisher's least significant difference test, $P \leq 0.05$).

Genotype and treatment	Cd	Ca	Mg	Mn	Zn
Leaves of plants grown in the presence of 3 μM $CdCl_2$ [μg g^{-1}(d.m.)]					
SGE	3.1 ± 0.8 ^{a}	332 ± 16 ^{a}	1382 ± 23 ^{a}	13 ± 1 ^{a}	29 ± 2 ^{a}
SGECd^t	7.7 ± 0.9 ^{b}	405 ± 18 ^{b}	1938 ± 58 ^{b}	17 ± 1 ^{b}	40 ± 2 ^{b}
Mesophyll protoplasts grown in the presence of 3 μM $CdCl_2$ [μg mg^{-1}(chlorophyll)]					
SGE	0.5 ± 0.2 ^{a}	115 ± 18 ^{a}	130 ± 27 ^{a}	0.8 ± 0.04 ^{a}	1.5 ± 0.2 ^{a}
SGECd^t	1.8 ± 0.1 ^{b}	150 ± 23 ^{a}	242 ± 3 ^{b}	1.4 ± 0.10 ^{b}	3.5 ± 0.1 ^{b}
Leaves of plants grown in the presence of 1 μM $CdCl_2$ [μg g^{-1}(d.m.)]					
SGE	2.3 ± 0.2 ^{a}	341 ± 9 ^{a}	1118 ± 43 ^{a}	14 ± 1 ^{a}	36 ± 1 ^{a}
SGECd^t	3.0 ± 0.5 ^{a}	362 ± 10 ^{a}	1317 ± 40 ^{a}	16 ± 1 ^{a}	39 ± 2 ^{a}
Leaves of plants grown in the presence of 4 μM $CdCl_2$ [μg g^{-1}(d.m.)]					
SGE	5.5 ± 0.6 ^{a*}	298 ± 8 ^{a*}	1049 ± 46 ^{a}	11 ± 1 ^{a*}	28 ± 1 ^{a*}
SGECd^t	12.1 ± 0.8 ^{b*}	355 ± 14 ^{b}	1247 ± 67 ^{b}	15 ± 1 ^{b}	33 ± 2 ^{b*}

Table 5. Distribution of elements between mesophyll protoplasts and the whole leaf in wild type (SGE) and mutant (SGECd^t) pea grown in the presence of 3 μM Cd. Calculations assume a 100 or 75 % distribution of Cd (see Materials and methods for calculation details).

Genotype	Relative share of elements in mesophyll protoplasts [%]			
	Cd	Mg	Mn	Zn
Cd solely localized in mesophyll protoplasts				
SGE	100	58	37	32
SGECd^t	100	53	34	37
75 % of leaf Cd localized in mesophyll protoplasts				
SGE	75	44	28	24
SGECd^t	75	40	26	28

showed substantial differences at 0.1 and 1 mM $CdCl_2$. To significantly inhibit ϕPSII in this short term experiment, higher Cd concentrations (in the mM range) were needed, exceeding those supplied in the nutrient solution (1 - 4 μM $CdCl_2$) almost a thousand-fold. This is not surprising since shoot metabolism is less sensitive than root growth, and in line with published data on Cd-treated leaf slices from *Arabidopsis* (Cho *et al.* 2003). Thus assessing the response of leaf tissue to Cd *in situ* revealed increased tolerance of the SGECd^t mutant grown in Cd-supplemented nutrient solution only, indicating that tissue tolerance is not entirely dependent on the whole plant. Instead, a molecular mechanism related to photosynthetic integrity appears to be induced by Cd, and this mechanism allows mutant tissue, pre-adapted to the presence of Cd in the nutrient solution, to tolerate higher Cd concentrations *in situ*. At higher Cd concentrations, the difference between wild type and mutant response was lost suggesting a limitation of this tolerance mechanism.

Cadmium content in leaves of SGECd^t treated with 3 and 4 μM Cd was about 2.5-times higher than SGE leaves (Table 4). Mesophyll protoplasts of SGECd^t treated with 3 μM Cd also contained 3.6-times more Cd. These data correspond well with previous reports on increased Cd content in intracellular leaf tissue and washing fluid, and mesophyll protoplasts of Cd-treated SGECd^t (Tsyganov *et al.* 2007, Belimov *et al.* 2015, 2016). Thus, foliar Cd accumulation did not prevent the mutant from maintaining active photosynthesis as indicated by unchanged ϕPSII. This observation confirmed our previous conclusion that SGECd^t is Cd insensitive (see references above). Although more detailed investigation is needed, it was proposed that increased water and nutrient transport from root to shoot is involved in Cd accumulation and tolerance to Cd of SGECd^t (Belimov *et al.* 2015, 2016). Reciprocal grafting experiments demonstrated that increased Cd tolerance required the presence of the trait in the roots (Belimov *et al.* 2018), indicating that root mechanisms are involved in photosynthetic adaptation to the toxic effects of Cd.

Nutrient (Ca, Mg, Mn, and Zn) content was higher in SGECd^t leaves compared to SGE (Table 4), consistent with observations that this mutant can counteract inhibition of nutrient uptake caused by Cd (Tsyganov *et al.* 2007, Belimov *et al.* 2016). Moreover, here we report for the first time that cell wall-free mesophyll cells (protoplasts) of SGECd^t mutant contained more Mg, Mn, and Zn after

treatments with Cd. Since Ca^{2+} was a constituent of the media used for protoplast preparation, the Ca^{2+} content measured in protoplasts may exceed the mesophyll content in the leaves *in vivo*. However, it should be noted, that plasma membrane Ca^{2+}-ATPases export Ca^{2+} from the cytosol to the apoplast, thus the Ca^{2+}influx is expected to be slow and low. Calcium in chloroplasts can regulate photosynthetic electron flow and light-dependent metabolism, trigger downstream signal transduction, and control xanthophyll cycle-dependent non-photochemical quenching (Brand and Becker 1984, Hochmal *et al.* 2015, Wang *et al*. 2019). Calcium may also mediate Cd-induced physiological or metabolic changes in plants as it is chemically similar to Cd (Huang *et al.* 2017). It may be hypothesized that the efficient Mg, Mn, and Zn homeostatic mechanisms of SGECd^t also maintained photosynthetic function. Efficient Mg homeostasis may *1*) prevent Cd substitution for Mg in chlorophyll, *2*) counteract the inhibition of photosynthetic enzyme activities (*e.g*. Rubisco having Mg^{2+} as a required factor for activation), and *3*) diminish oxidative stress (Krupa *et al.* 1995, Siedlecka *et al.* 1998, Sandalio *et al.* 2001, Shaul 2002). Manganese is required to form the Mn-Ca-cluster in PS II, which catalyses the water-oxidation process within the oxygen-evolving complex (Kalstyan *et al.* 2012, Yruela 2013). The important micronutrient Zn is involved in protecting plants against oxidative stress, *e.g*., by being part of the catalytic centre of Cu/Zn superoxide dismutase (Cakmak 2000). Oxidative stress is associated with Cd toxicity in various plants including pea (Sandalio *et al.* 2001, Metwally *et al*. 2005). Zn is also co-factor of chloroplastic β-carbonic anhydrase and Zn-finger proteins located in thylakoid membranes (Kimber and Pai 2000, Yruela, 2013). Although there were no genotypic differences in the contents of Fe, K, P, and S in leaves and in protoplasts (data not shown), both Cd-untreated pea genotypes had similar leaf content of these elements (data reported in Tsyganov *et al.* 2007 and Belimov *et al.* 2016).

Based on the nutrient content in leaves and mesophyll protoplasts, elemental distribution can be estimated (Table 5). Cadmium was the element with the highest relative abundance in the mesophyll protoplasts. Dividing leaf Cd content by protoplast Cd content gave a factor of 6.2 in SGE and 4.3 in SGECd^t. The same approach applied to the other elements indicates that relative mesophyll compartmentation of Mg and Mn is lower in SGECd^t than in SGE. However, this is compensated by the higher total element content in SGECd^t. Interestingly, the distribution was reversed for Zn^{2+} such that more was in the mesophyll of SGECd^t than SGE. These results suggest that ionic and Cd homeostasis may be affected by both root transport processes (Belimov *et al.* 2018) but also in the leaf mesophyll processes that affect vacuolar deposition. The latter property can play an important role in detoxifying Cd to allow stable photosynthesis in the cytoplasm of SGECd^t.

Conclusions

The results showed that when leaf tissue was taken from plants grown in Cd-containing nutrient solution (but not those previously unexposed to Cd) and incubated *in vitro* with Cd, SGECd^t revealed an increased tolerance of the photosynthetic quantum yield ϕPSII. We suggest that the tolerance mechanism is induced only in the presence of Cd. The improved acclimation ability of SGECd^t leaves likely is related to the higher foliar nutrient content, allowing better functioning of the photosynthetic apparatus and maintenance of photosynthetic integrity by counteracting substitution of nutrient elements and development of leaf damage caused by Cd.

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