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Feedback inhibition by thiols outranks glutathione depletion: a luciferase-based screen reveals glutathione-deficient γ -ECS and glutathione synthetase mutants impaired in cadmium-induced sulfate assimilation

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Summary

Plants exposed to heavy metals rapidly induce changes in gene expression that activate and enhance detoxification mechanisms, including toxic-metal chelation and the scavenging of reactive oxygen species. However, the mechanisms mediating toxic heavy metal-induced gene expression remain largely unknown. To genetically elucidate cadmium-specific transcriptional responses in Arabidopsis, we designed a genetic screen based on the activation of a cadmium-inducible reporter gene. Microarray studies identified a high-affinity sulfate transporter (*SULTR1;2*) among the most robust and rapid cadmium-inducible transcripts. The *SULTR1;2* promoter (2.2 kb) was fused with the firefly luciferase reporter gene to quantitatively report the transcriptional response of plants exposed to cadmium. Stably transformed luciferase reporter lines were ethyl methanesulfonate (EMS) mutagenized, and stable M_2 seedlings were screened for an abnormal luciferase response during exposure to cadmium. The screen identified non-allelic mutant lines that fell into one of three categories: (i) super response to cadmium (*SRC*) mutants; (ii) constitutive response to cadmium (*CRC*) mutants; or (iii) non-response and reduced response to cadmium (*NRC*) mutants. Two *nrc* mutants, *nrc1* and *nrc2*, were mapped, cloned and further characterized. The *nrc1* mutation was mapped to the γ-glutamylcysteine synthetase gene and the *nrc2* mutation was identified as the first viable recessive mutant allele in the glutathione synthetase gene. Moreover, genetic, HPLC mass spectrometry, and gene expression analysis of the *nrc1* and *nrc2* mutants, revealed that intracellular glutathione depletion alone would be insufficient to induce gene expression of sulfate uptake and assimilation mechanisms. Our results modify the glutathione-depletion driven model for sulfate assimilation gene induction during

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cadmium stress, and suggest that an enhanced oxidative state and depletion of upstream thiols, in addition to glutathione depletion, are necessary to induce the transcription of sulfate assimilation genes during early cadmium stress.

Keywords

glutathione biosynthesis; heavy metal; γ-glutamylcysteine synthetase; metabolite-based cloning; phytochelatins; *Arabidopsis thaliana*

Introduction

Toxic metals such as lead, cadmium (Cd), mercury and the metalloid arsenic can accumulate in soils and water to levels that are detrimental to human and environmental health. Many human disorders have been attributed to the ingestion of heavy metals, including learning disabilities in children, dementia, impairment of bone metabolism and increased cancer rates (Tong *et al*., 2000; Allen *et al*., 2002; Aschner and Walker, 2002; Ohta *et al*., 2002; Yu *et al*., 2002; Waisberg *et al*., 2003; Heck *et al*., 2009; Satarug *et al*., 2010). Food crops are a major source of heavy metal intake in humans, which has prompted interest in understanding how plants take up, detoxify and retain heavy metals. In addition, plants hold the potential for the development of a cost-effective approach for the removal and remediation of heavy metalladen soils and water through the use of metal-hyperaccumulating plants (phytoremediation) (Raskin *et al*., 1994; Dushenkov *et al*., 1995; Salt *et al*., 1995, 1998; Clemens, 2006).

Metal trafficking, both within the cell and between different tissues, often requires the use of metal ligand molecules such as citrate, nicotianamine, glutathione (GSH) and phytochelatins (PCs) (Lee *et al*., 1978; Grill *et al*., 1985; Howden *et al*., 1995; Kramer *et al*., 2000; Sanchez-Fernandez *et al*., 2001; Klein *et al*., 2002; Richau *et al*., 2009; Mendoza-Cozatl *et al*., 2011). Glutathione is a crucial molecule required for the synthesis of PCs, which detoxify mercury, Cd and the metalloid arsenic. PCs are small glutathione polymers synthesized in the cytosol (Grill *et al*., 1985; Clemens *et al*., 1999, 2001; Ha *et al*., 1999; Vatamaniuk *et al*., 1999, 2001). PCs bind highly toxic heavy metals and metalloids, and transport them into the vacuoles by ABC transporters (Li *et al*., 2004; Chen *et al*., 2006; Mendoza-Cozatl *et al*., 2010; Song *et al*., 2010). Glutathione and PCs have been shown to undergo long-distance transport of Cd through the phloem, but the identities of these transporters remain unknown (Gong *et al*., 2003; Chen *et al*., 2006; Mendoza-Cozatl *et al*., 2008). Thus, exposure to heavy metals can rapidly deplete glutathione levels and create an extremely high demand for glutathione.

At the transcriptional level, heavy metal exposure elicits a robust gene expression response in plants (Herbette *et al*., 2006; Weber *et al*., 2006). For instance, Cd exposure rapidly depletes cells of GSH, which in turn induces transcripts that encode sulfate uptake, sulfate assimilation and glutathione biosynthesis mechanisms (Lee and Leustek, 1999; Nocito *et al*., 2006; Davidian and Kopriva, 2010). These findings have led to the development of a metabolite demand-driven model for the regulation of sulfate assimilation and glutathione biosynthesis in which heavy metal-induced GSH depletion induces gene expression

(Vauclare *et al*., 2002; Kopriva, 2006). However, the molecular mechanisms that trigger rapid changes in gene expression following heavy metal exposure in plants remain unknown.

To uncover the molecular and genetic mechanisms that mediate rapid Cd-induced gene expression in Arabidopsis, we have pursued Cd-induced microarray experiments and a forward genetic screen to identify mutants with altered responses to Cd exposure, using a Cd-inducible promoter driving the expression of the firefly luciferase gene. Unexpectedly, two of the mutants showing a dramatically decreased Cd response are impaired in steps upstream of GSH synthesis. HPLC-MS analyses of thiol compounds suggest that upstream thiols and an oxidative redox state functions in the induction of sulfate uptake genes during Cd exposure, and not during GSH depletion alone. Characterization of the transcriptional response to Cd in these mutants revealed a new level of regulation (hierarchical regulation) of sulfur assimilation signaling and glutathione biosynthesis in response to Cd exposure in plants.

Results

Identification of a rapid cadmium-inducible promoter

To identify genes in Arabidopsis that are highly and rapidly induced by Cd, we performed oligonucleotide chip-microarray experiments (Affymetrix, ATH1) on 1-week-old Arabidopsis seedlings exposed to 200 μ_M $CdCl_2$ for 6 h (Table S1). Cd-inducible transcripts were identified (Table S1) and transcriptional activation following Cd exposure was confirmed for six strongly induced transcripts by RT-PCR (Figure 1a). Nine promoter-luciferase constructs containing 2.2-kb promoter fragments of the cadmium-inducible genes were introduced into Arabidopsis (Col-0), and stable T_3 homozygous seedlings were analyzed for cadmium-induced luminescence. Luciferase (LUC) reporter lines carrying the high-affinity sulfate transporter *SULTR1;2* promoter (*pSULTR1;2*) showed a quantitative and highly reproducible luciferase response to Cd, and one line (line A) was chosen for mutagenesis (Figure 1b). This *pSULTR1;20*::*LUC* reporter line will henceforth be referred to as the control reporter line or parental line.

pSULTR1;2::LUC is induced by cadmium, arsenate and copper, but not by exogenous reactive oxygen species

The dynamic response of 4 reporter lines was analyzed over a 12-h period following Cd exposure (Figure 1b,c). Luciferase activity was highest in roots, and the induction was evident after 1 h of Cd exposure, reaching a maximum after 3 h of exposure (Figure 1b) and decreasing steadily to half of the maximal induction after 12 h of Cd exposure (Figure 1c). To determine if the *SULTR1;2* promoter is induced broadly by metals or exogenous reactive oxygen species (ROS), luciferase induction was measured following exposure to arsenate, copper, aluminum, nickel, cobalt and the ROS-inducing agent paraquat (Figure 1d–i). Figure 1 shows that Cd (Figure 1c), arsenate (Figure 1d) and copper (Figure 1e) elicit a strong transcriptional response, whereas the remaining metals and paraquat showed limited or no induction during the 12-h exposure period. These results suggest that *SULTR1;2* is not broadly induced by oxidative stress and that the *SULTR1;2* induction line is a suitable

parental line for a forward genetic screen to identify mutants with an impaired Cd-induced transcriptional response.

Seeds of the control reporter line were ethyl methanesulfonate (EMS) -mutagenized (approximately 6000 seeds), and 60 000 M_2 seedlings were screened for altered luciferase induction after 6 h of Cd exposure. Putative mutants were selected and the altered luciferase response was confirmed in M_3 seedlings (Figure 2). Mutants were classified into one of three different groups: (i) *constitutive response without Cd* (*CRC*) mutants, showing a constitutive luciferase induction (Figure 2b) without being exposed to Cd; (ii) *super response to Cd* (*SRC*) mutants, which showed higher luciferase activity compared with the control reporter lines following Cd exposure (Figure 2c); (iii) *non-response or reduced response to Cd* (*NRC*) mutants, which failed to induce strong luciferase activity after Cd exposure (Figure 2d,e).

Glutathione-deficient mutants show reduced luciferase induction during Cd exposure

We focused on characterization of two recessive non-response mutants, designated *non-response to cadmium 1* and *2* (*nrc1* and *nrc2*), which are Cd sensitive and have short roots when grown in the presence of Cd. Figure 2(d,e) shows the luciferase phenotype of the *nrc1* and *nrc2* mutants. To validate the decreased luciferase response of the *nrc1* and *nrc2* mutants, RT-PCR analysis of the native *SULTR1;2* gene was performed in the control and in the *nrc1* and *nrc2* mutants. Figure 2(f,h) shows that the induction of the *SULTR1;2* transcript in *nrc1* was severely decreased compared with the control reporter line (Figure 2f), but only moderately decreased in the *nrc2* mutant (Figure 2h). The reduced size of *nrc2* seedlings probably contributed to the difference between the measured decreases in luciferase response in the *nrc2* mutant, with a moderate decrease in *Sultr1;2* transcript level in the *nrc2* mutant. Thus, the *nrc*1 mutant is a strong non-response mutant, whereas the *nrc2* mutant, which retains some *Sultr1;2* induction, is more accurately described as a reduced response mutant. Root elongation experiments on plates containing 20 μM $CdCl_2$ showed that *nrc1* and *nrc2* seedlings are Cd hypersensitive (Figure 2g,i). Crosses between *nrc2* and *nrc1* showed that they are non-allelic.

The organic thiols cysteine, γ-glutamylcysteine (γ-EC) and GSH are known to be key metabolites required for the production of PCs that mediate Cd detoxification. Therefore, we analyzed the metabolic thiol profile of the *nrc1* and *nrc2* mutants by fluorescence HPLC coupled to a mass spectrometer (HPLC-MS) (Figure 3a-f). After exposure to 20 μM Cd for 48 h, GSH levels were decreased in both the *nrc1* (44.8 ± 2.31 nmol GSH per g fresh weight) and the *nrc2* (94.9 ± 8.22 nmol GSH per g fresh weight) mutants compared with parental controls (126.2 nmol GSH per g fresh weight; Figure 3a-c,f). Conversely, cysteine levels in the *nrc1* (66.6 ± 8.19 nmol Cys per g fresh weight) and *nrc2* (51.3 ± 8.93 nmol Cys per g fresh weight) mutants in the presence of Cd were elevated compared with parental controls (11.8 ± 0.71 nmol Cys per g fresh weight; Figure 3a-d). Interestingly, γ-EC levels were decreased following Cd exposure in the *nrc1* mutant (2.30 ± 0.40 nmol γ-EC per g fresh weight), and were elevated in the *nrc2* mutant (431.7 ± 72.7 nmol γ-EC per g fresh weight), compared with parental controls (8.57 ± 0.25 nmol γ-EC per g fresh weight; Figure 3a-c,e).

Physical mapping and characterization of the *nrc1* and *nrc2* mutants

Our HPLC-MS findings suggest that *nrc1* inefficiently converts cysteine into γ-EC, the precursor of GSH and PCs. Initial rough mapping using an F_2 population of a *nrc1* × Landsberg *erecta* (*Ler*) backcross located the mutation on chromosome 4, between the nga1107 and ciw7 markers (Figure 4a). Based on the thiol profile of *nrc1*, candidate genes involved in sulfur assimilation and GSH synthesis from this mapping region were PCR-amplified and sequenced. The locus At4g23100 contained a single C→T mutation in the fourth exon causing a Pro →Leu (P214L) change in the amino acid sequence of γ-EC synthetase (γ-ECS), a key enzyme in glutathione biosynthesis (Figure 4a).

To further determine whether this mutation in γ-ECS was responsible for the *nrc1* phenotype, *nrc1* was crossed into the previously characterized γ-ECS allele, *cad2-1* (Howden *et al*., 1995) (Figure 4b). F_1 seedlings from the reciprocal crosses between the recessive *nrc1* and *cad2-1* mutants were Cd hypersensitive, as determined by root elongation assays in the presence of cadmium (Figure 4b). In contrast, in the presence of Cd, seedlings from crosses of wild-type Col-0 (WT) and *nrc1* or *cad2-1* were not Cd hypersensitive (Figure 4b). These results show that *nrc1* is allelic to *cad2-1*.

To further compare the *nrc1*allele with the *cad2*-1allele, we compared the γ-ECS activity in protein extracts obtained from the two mutants versus the activity of γ-ECS in WT extracts. Using HPLC-MS to determine the initial rates of activity of γ-ECS, we determined that WT extracts synthesizes γ-EC at a rate of 74pmoles –SH min)$^{-1}$ mg protein)$^{-1}$, whereas *nrc1* and *cad2-1* synthesize γ-EC at a rate of 13 pmoles –SH min^{-1} mg protein^{-1} (17.56% of the WT rate) and 14 pmoles –SH min^{-1} mg protein^{-1} (18.91% of the WT rate), respectively (Figure 4c). These results suggest that the point mutation in *nrc1*is as severe as the 6-bp deletion found in the *cad2-1* mutant. To further confirm the causative mutation in the *nrc1* mutant, we expressed the genomic *γ-ECS* gene, beginning with the start codon and excluding the 5′ and 3′ untranslated regions (UTRs), ectopically behind the CaMV *35S* promoter in the *nrc1* mutant background. Three independent transformant lines (γ-ECS-Comp1–γ-ECS-Comp3) were selected and T_2 seedlings from these lines were used for root elongation studies and fluorescence HPLC-MS. Ectopic expression of *γ-ECS* in the *nrc1* mutant background rescued the Cd-sensitive root growth phenotype of the *nrc1* mutant (Figure 4d), and greatly decreased the cysteine accumulation phenotype (Figure 4e). Taken together, these results support the conclusion that the identified mutation in *γ-ECS* is the causative mutation in the *nrc1* mutant.

Our HPLC-MS results also suggest that *nrc2* inefficiently converts γ-EC into GSH (Figure 3a,c,f). Genetic mapping using an F_2 population of an *nrc2* × Landsberg *erecta* (L*er*) backcross located the mutation on chromosome 5, between the T21B4 and F15F15 markers (Figure 5a). Based on the thiol profile of *nrc2*, candidate genes involved in sulfur assimilation and GSH synthesis from this mapping region were PCR amplified and sequenced. The locus At5g27380 contained a single C → T mutation in the 10th exon, causing an Ala → Val (A404V) change in the amino acid sequence of glutathione synthetase (*GS*), the final enzyme in glutathione biosynthesis (Figure 5a).

No previous viable mutation in the Arabidopsis *GS* gene has been identified, and *GS* T-DNA insertion mutants have been shown to be seedling lethal (Pasternak *et al*., 2008). Therefore, to determine whether the *GS* point mutation was responsible for the *nrc2* mutant phenotype, the genomic *GS* gene, beginning from the start codon and excluding the 5′ and 3′ UTR regions, was ectopically expressed behind the CaMV *35S* promoter in the *nrc2* genetic background. Root elongation using T_2 transformant seedlings from three independent transformant lines (GS-Comp1–GS-Comp3) grown on 20 μM Cd confirmed that ectopic expression of the *GS* gene complemented the Cd-sensitive phenotype in the *nrc2* mutant (Figure 5b). Furthermore, 21-day-old soil-grown seedlings appeared less chlorotic than the *nrc2* mutant (Figure S1). Subsequent HPLC-MS analyses of WT, *nrc2* and T_2 seedlings treated with 20 μM Cd show that γ-EC levels were drastically decreased in the complemented lines (Figure 5c). To determine whether this increase in Cd tolerance was an artifact of ectopic expression of GS, we also transformed WT Col-0 with the same GS construct, and selected independent T_2 transformant lines (GS-OX1–GS-OX5). RT-PCR analysis was performed to select lines showing an increase in *GS* transcript (Figure 5d) relative to the WT. We then performed root elongation experiments on 20 μM Cd using these overexpression lines and confirmed that ectopic expression of *GS* does not increase Cd tolerance compared with wild-type lines (Figure 5e). These findings together provide strong evidence that the *nrc2* phenotype is caused by the identified recessive point mutation in *GS*.

Sultr1;2 induction is repressed, even when GSH is depleted

The transcriptional upregulation of sulfate assimilation genes has been described as being part of the plant response to GSH depletion (e.g. PC synthesis during Cd exposure causing GSH depletion; Rouached *et al*., 2008; Saito, 2004). However, in contrast to this model, the *nrc1* and *nrc2* mutants showed clear GSH depletion (Figure 3d), but failed to produce a strong induction of the SULTR1;2 promoter-driven luciferase reporter (Figure 2d,e) and the native *Sultr1;2* mRNA to wild-type levels after cadmium exposure (Figure 2f,g). These findings point to an alternative hypothesis that the over-accumulation of thiol compounds, either as cysteine (Figure 3d) or γ-EC (Figure 3e), represses the induction of *SULTR1;2* gene expression during Cd exposure in these mutants, even though GSH levels are reduced (Figure 3f). A possible mechanism mediating this response may be that the thiol-dependent cellular redox state also contributes to the Cd-induced gene expression of *Sultr1;2*. To test this hypothesis, we conducted thiol feeding experiments in the presence of cysteine or γ-EC added to the growth medium. Figure 6a shows that the addition of cysteine or γ-EC to the growth media attenuates the induction of *SULTR1;2* gene expression in response to Cd. From the above experiments, however, it was unclear whether the addition of cysteine or γ-EC repressed the luciferase activity by extracellular Cd chelation, metabolite repression or altered cellular redox state. Therefore, we analyzed whether the addition of cysteine, GSH, DTT (a non-physiological thiol) and the non-thiol reducing agent, butylated hydroxyanisole (BHA, which is not known to chelate Cd; Gulcin *et al*., 2003), altered the Cd-dependent induction of *SULTR1;2* gene expression. As shown in Figure 6b, feeding the non-physiological reducing agents DTT and BHA repressed Cd-induced luciferase activity (Figure 6b) to a similar degree as the metabolites cysteine or GSH (Figure 6b). These results are consistent with a hypothesis where a reducing cellular environment in the *nrc1* and *nrc2* mutants, caused by cysteine or γ-EC over-accumulation, represses Cd-induced gene

expression despite low GSH levels in these mutants. Thus, a reducing cellular environment would have hierarchical control, repressing *SULTR1;2* gene induction (Figure 6c). In this experiment, reducing compounds, including the non-physiological reducing agents DTT and BHA, prevent Cd-induced signal transduction, despite the depletion in GSH levels caused by PC production. Furthermore, our results indicate that an increased level of reducing thiols, including cysteine or γ-EC, inhibits Cd-induced *Sultr1;2* gene expression *in vivo*, even when GSH levels are depleted (Figure 6c).

Discussion

A luciferase-based genetic screen was devised using Cd-dependent microarray analysis and the Cd-inducible *Sultr1;2* promoter. In Arabidopsis the *Sultr1;2* transcript is induced by Cd (Rouached *et al*., 2008) (Figure 1). Microarray analysis (Table S1), RT-PCR analysis (Figures 1a and 2a) and luciferase imaging (Figure 1b,c) demonstrate that the 2.2-kb *SULTR1;2* promoter fragment is a rapid and robust reporter of Cd exposure. Furthermore, we show that the *SULTR1;2* promoter fragment is induced by a well-defined set of metals and metalloids (arsenic), but is induced less well by ROS-inducing agents, such as paraquat (Figure 1c–i).

Our Cd-inducible reporter screen allowed us to identify mutants with decreased, constitutive and increased activity of the reporter gene (Figure 2). These classes of mutants suggest that sulfate uptake in Arabidopsis is regulated by antagonistic transcriptional activators and repressors. To date, one transcriptional regulator of *SULTR1;2*, SLIM1, has been reported and is regulated under sulfur limiting conditions (Maruyama-Nakashita *et al*., 2006). It remains unknown whether SLIM1 functions in the Cd response, and none of our strong *nrc* mutants mapped to the *SLIM1* gene. The components of the Cd-dependent transcriptional signaling pathway in Arabidopsis remain unknown. Isolation and characterization of the Arabidopsis *nrc* mutants was pursued to advance our understanding of the levels of genetic and mechanistic regulation of the sulfate assimilation pathway that occurs during Cd exposure.

Current model of sulfur homeostasis and GSH depletion during Cd stress

Glutathione is known to be important for mitigating stress as the GSH-deficient mutants *cad2-1*, *pad2*, *rax1-1* and *zir1* have all been identified by their sensitivity to abiotic or biotic stresses (Cobbett *et al*., 1998; Ball *et al*., 2004; Parisy *et al*., 2007; Shanmugam *et al*., 2011). Another GSH-deficient mutant, *rml*, was identified as lacking a root meristem and having a severe growth and developmental phenotype (Vernoux *et al*., 2000). These mutants display GSH depletion of various degrees, with the *rml* mutant having the least GSH (approximately 3% of wild-type levels) and *rax1-1* having the highest GSH levels (approximately 50% of wild-type levels) (Vernoux *et al*., 2000; Ball *et al*., 2004; Shanmugam *et al*., 2011). Whereas the severity of these mutations is typically linked to the degree of GSH depletion, Shanmugam *et al*. (2011) have recently shown by systematically analyzing the iron-induced zinc tolerance of each of these mutants that a threshold level of GSH is required for some phenotypes. These results suggest that the phenotypes observed in these mutants may not be linearly correlated with GSH levels alone. Furthermore, the *nrc1* × cad2-1 F_1 plants (Figure

4b) showed slightly longer root growth than either the *nrc1* mutant or the *cad2-1* mutant alone. This suggests that when the two mutant alleles are expressed together, despite having similar GSH content, the *nrc1* × *cad2-1* F_1 crosses are slightly less sensitive to Cd. One explanation for this observation is that in the *nrc1* × *cad2-1* cross, the γ-ECS dimer is more functional than in the *nrc1* or *cad2-1* offspring (Hothorn *et al*., 2006; Gromes *et al*., 2008). This would be consistent with recent findings showing that the regulation of *γ-ECS* in plants is complex and occurs at both the transcriptional and post-transcriptional levels (Hothorn *et al*., 2006; Gromes *et al*., 2008).

The current model for sulfur homeostasis in plants proposes that GSH, the most abundant organic thiol in plants, is a strong negative regulator of both sulfate assimilation and cysteine biosynthesis. Glutathione is known to repress the expression and activity of high-affinity sulfate transporters, ATP sulfurylase and APS reductase (Kopriva, 2006). Cellular GSH levels decrease during Cd stress as a result of PC production (Rauser, 1995). This GSH depletion causes an increase in cellular GSH demand, increasing the transcription of sulfate uptake-related genes (i.e. *SULTR1;2*) (Kopriva and Rennenberg, 2004; Kopriva, 2006) (Figure 1). This model argues that as sulfate assimilation restores thiol levels, GSH represses sulfate uptake and assimilation genes (Kopriva and Rennenberg, 2004; Kopriva, 2006). Tight regulation of GSH synthesis is needed because of the high reactivity yet essential nature of GSH. Feedback regulation allows the rapid activation of sulfate assimilation during a sudden decrease in GSH levels. This model accounts for many observations, but it assumes that GSH is the major regulator of sulfate assimilation during Cd stress. Growing evidence indicates that cysteine and H_2S are also potent repressors of sulfate transporters; however, it is unclear if cysteine and H_2S repression are direct or mediated through an increase in GSH (Lappartient and Touraine, 1996; Vauclare *et al*., 2002; Maruyama-Nakashita *et al*., 2004). Here, by isolating and characterizing mutations that insulate variations in cysteine and γ-glutamylcysteine from changes in GSH concentration, we show the key role of sulfur-containing compounds synthesized prior to GSH production in repressing Cd-induced gene expression (Figure 5c).

nrc mutants reveal cysteine and γ-EC as potent SULTR1;2 repressors

The identification and characterization of the *nrc1* and *nrc2* mutants suggest that another level of Cd-induced gene expression regulation exists. The *nrc1* mutant is GSH deficient but accumulates high levels of cysteine (Figure 3b,d,e), whereas the *nrc2* mutant has decreased GSH levels but accumulates high levels of γ-EC (Figure 4c–e). According to the current model, the GSH status of these mutants should induce *SULTR1;2*, particularly after Cd exposure. Thus, we would expect these mutants to be constitutive or super-response mutants (Figure 2). However, in the *nrc1* mutant, Cd-induced *SULTR1;2* gene expression was strongly repressed, and in the *nrc2* mutant, Cd-induced *SULTR1;2* gene expression was decreased (Figure 2d,e).

We hypothesized that the aberrant accumulation of thiol compounds in the *nrc1* (cysteine) and *nrc2* (γ-EC) mutants also caused a reducing redox environment during Cd exposure, leading to a downregulation of *SULTR1;2* (Figure 6c). Our findings indicate that this reducing cellular state may contribute as a repression mechanism of sulfate assimilation

genes in response to Cd stress. Evidence to support this was obtained by feeding experiments with physiological (cysteine and GSH) and non-physiological (DTT) reducing agents, as well as reducing agents not known to chelate Cd (BHA) (Figure 6b). Interestingly, feeding cysteine, γ-EC or GSH to the pSULTR: :LUC parental line lowered the Cd-induced luciferase response (Figure 6a,b). Furthermore, feeding non-physiological reducing agents such as DTT and BHA also decreased the *Sultr1;2* induction during Cd exposure (Figure 6b). These results, together with our thiol profiling of isolated genetic mutants, are inconsistent with a solely GSH depletion-driven model, and point to a model where GSH depletion, upstream thiol concentrations and an oxidized cellular redox state are required to induce sulfate assimilation in Arabidopsis in response to Cd stress (Figure 6c). The elevated cysteine or γ-EC thiol levels and reducing cellular conditions in the *nrc1* and *nrc2* mutants are proposed to repress the induction of sulfate assimilation genes, despite the low GSH concentrations in the mutants (Figure 3d). Thus, our results suggest that sulfate assimilation in Arabidopsis is controlled in a hierarchical manner by upstream thiols, the redox state of the cell and the concentration of GSH (Figure 6c). However, oxidative stress alone is not sufficient for mediating Cd-induced *SULTR1;2* expression. Indeed, oxidizing agents such as paraquat (Figure 1f) and H_2O_2 did not induce *SULTR1;2* expression as highly or rapidly as Cd (Figure 1), presumably because they do not cause a decrease in organic thiols despite altering the cellular redox state (Figure 6c).

Regulation of sulfate assimilation and glutathione biosynthesis

The *Sultr1;2* transcript is induced by several stresses, including Cd, attack by pathogens and sulfur deprivation (Maruyama-Nakashita *et al*., 2006). The *SULTR1;2* promoter was previously used as a reporter gene to screen for mutants unable to induce genes regulated during sulfur starvation (Maruyama-Nakashita *et al*., 2005). This screen led to the identification of SLIM1 (EIL3), an ethylene insensitive-like transcription factor that regulates the expression of *SULTR1;2* and of genes that mediate glucosinolate synthesis (Maruyama-Nakashita *et al*., 2006). The SLIM1 protein has been proposed to be a transcriptional activator under conditions of sulfur starvation (Segarra *et al*., 2009). Presently, there is no direct evidence supporting its role as a transcriptional activator of the *SULTR1;2* promoter. To date it is not known whether SLIM1 directly regulates the expression of *Sultr1;2* or whether its function is independent of *O*-acetylserine (a precursor of cysteine) and GSH concentrations (Maruyama-Nakashita *et al*., 2006). In summary, the isolation and characterization of the non-response to Cd mutants *nrc1* and *nrc2* points to a new model (Figure 6c) for the regulation of gene expression in response to Cd stress, in which several criteria are necessary for Cd-induced gene expression: (i) GSH depletion; (ii) depletion of upstream thiol levels; (iii) an oxidative cellular redox state, which together control the Cd-induced transcription of *SULTR1;2*.

Experimental procedures

Arabidopsis accessions

The WT *Arabidopsis thaliana* ecotypes used for mapping were Columbia (Col-0) and Landsberg *erecta* (L*er*-0). The *nrc1* and *nrc2* mutants are in the Col-0 genetic background, and the transformant line pSULTR1;2: :LUC is also in the Col-0 genetic background.

Plant growth conditions

Seeds were sterilized and plated on plates containing quarter-strength MS standard medium (M5519; Sigma-Aldrich, http:// www.sigmaaldrich.com), 1 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES), 1% phytoagar (Duchefa, http://www.duchefa.com) and the pH adjusted to 5.6 with 1.0 M KOH (Maser *et al*., 2002; Lee *et al*., 2003). Sterilized nylon mesh with a 200-μm pore size (Spectrum Labs, http://www.spectrumlabs.com) was placed on the surface of the media prior to sowing the seeds. The seeds were then stratified with cold treatment at 4°C for 48 h, and grown under growth room conditions for 5 days (300 μmol m^{-2} s^{-1},70% Hr, 16-h light at 21°C/8-h dark at 18°C)(Sung *et al*., 2007). Seedlings were then transferred to quarter-strength MS, 1 mM MES and 1% agar plates containing 20 μM $CdCl_2$.

Construction of cadmium-response luciferase reporter line

Nine Arabidopsis promoters (2.2 kb before the ATG) from genes found to be induced by cadmium were cloned into the pPZPXomegaL+ vector (kindly provided by Dr. Steve Kay) with *Bam*H1 and/or *Hind*III restriction digestion. The correct orientation of the promoters was confirmed by sequencing the cloned promoter regions in the vector. These nine luciferase constructs were transformed into Arabidopsis and the resulting T_3 homozygous transgenic luciferase plant lines were tested for induction of luciferase protein by monitoring bioluminescence for up to 12 h after exposure to Cd. Of the nine transgenic luciferase reporter lines, several reporter lines containing the promoter of a high-affinity sulfate transporter gene (*SULTR1;2*) showed the most reproducible induction of luciferase, and were hence selected as the reporter lines for the cadmium transcriptional response screening.

Mutant isolation by luciferase luminescence imaging

Homozygous T_3 seeds of a *pSULTR1;2* reporter line were mutagenized with 0.25% EMS for 14 h. The survival of 50% of the mutagenized seeds was confirmed as an indicator of adequate mutagenesis. M_1 seeds were bulk harvested from approximately 6000 M_0 plants and approximately 200 000 M_1 seeds were screened for altered luminescence patterns in response to cadmium treatment (200 μM for 6 h). This is between two and four times more than the minimum M_1 population required to find a mutation in any given G:C pair (Jander *et al*., 2003). For luciferase imaging, the protocol described by Chinnusamy *et al*. (2002) was followed with the following modifications (Chinnusamy *et al*., 2002). Seedlings were grown for 5 days horizontally on 36-μm Nitex mesh (Small Parts, Seattle, WA, USA) before being presprayed with 5 mM luciferin (Promega, http://www.promega.com) 6 h before being transferred to either control or treatment plates in order to minimize non-specific luminescence. After transfer the seedlings were subjected to another spraying of 5 mM luciferin and incubated for a period of time (as indicated in the results section) before being imaged using a BERTHOLD NightOWL LB981 imaging system (EG&G Berthold, http:// www.berthold.com). A 2-min exposure time was used for capturing the bioluminescent images. Luminescence was quantified using NIGHTOWL.

Total RNA isolation and RT-PCR analysis

Plant materials were flash frozen into liquid nitrogen immediately after treatments. Plant materials were ground using a pre-chilled mortar and pestle. A 100-mg portion of ground plant powder was used to extract total RNA by using a commercial RNA extraction kit (Qiagen, http://www.qiagen.com) (Sunarpi *et al*., 2005). The quantity and quality of total RNA was recorded using spectrophotometry and gel electrophoresis. A 5-μg portion of total RNA was treated with DNase1 (Ambion, now Invitrogen, http://www.invitrogen.com) to remove DNA contamination from total RNA samples. Prior to the reverse-transcription reaction, DNase-treated total RNA was heated to 65°C for 10 min and immediately cooled down in ice to minimize the secondary structures of total RNAs. A 1-μg portion of total RNA was reverse transcribed with a *Not*I-d$(T)_{18}$ primer using a First Strand cDNA kit (GE Healthcare, http:// www.gehealthcare.com) for 60 min at 37°C. Reverse-transcribed cDNA was subjected to PCR to amplify the expression signal of each gene, with the following typical conditions: initial denaturation of cDNA/RNA and inactivation of reverse transcriptase at 95°C for 5 min, then DNA amplification with 25–40 cycles of 95°C for 15 s, 52°C for 15 s, 72°C for 1 min, then a final extension at 72°C for 5 min using an MJ Research PTC 100 Thermal Cycler (GMI, http:// www.gmi-inc.com). As a loading control, elongation factor-1α (EF-1α) mRNA was analyzed. Table S2 contains a complete list of all primers used in this study.

Thiol measurements by fluorescence HPLC

Thiol-containing compounds in plant samples, including cysteine, γ-EC, GSH and PCs, were analyzed using fluorescence detection HPLC, as described by Fahey and Newton (1987). To analyze the levels of thiol compounds produced by plants in response to treatment, plants were grown on minimal growth media plates for 5 days then transferred to fresh media plates containing 200 μM cadmium. In order to minimize the oxidation of thiol compounds during the extraction, plant seedlings were flash-frozen in liquid nitrogen, and then ground and extracted as previously described (Sung *et al*., 2009). The peaks of thiol compounds were identified by coupled parallel mass spectrometry measurement, as previously described (Chen *et al*., 2006), and quantified using XCALIBUR (Thermo Scientific, http:// www.thermoscientific.com). To identify the peptides from plant extracts, PC2, PC3 and PC4 standards were synthesized on a MILLIGEN 9050 PepSynthesizer (Millipore, http://www.millipore. com) using Fmoc-Glu-OtBu (Bachem, http://www.bachem.com). Other thiol standards, such as glutathione, cysteine, γ-EC and NAC, were purchased from Sigma-Aldrich. All reported thiol quantities are means of between three and nine biologically independent samples, and error bars indicate the standard error of the mean (SEM).

Plant growth conditions and cadmium treatment

For plate-based assays, including heavy metal treatments, luciferase luminescence assay and plant growth for RT-PCR analysis, seeds were germinated on nylon mesh (Spectrum Laboratories Inc., http://www.spectrumlabs.com) on quarter-strength MS media and grown for 1 week at 22°C, 75% humidity, with a 16-h light/8-h dark photoperiod regime at approximately 75 μmol m^{-2} s^{-1} light intensity in a Conviron growth chamber (Controlled

Environments Inc., http://www.conviron.com). Seedlings on nylon mesh were transferred either to treatment or control plates and incubated for up to 12 h, as previously described (Sung *et al*., 2009).

Feeding experiments

We performed feeding experiments using the control pSULTR1;2: :LUC reporter line (Figure 1). We exposed 5-day-old seedlings to 100 μM Cd, 100 μM Cd + 2500 μM Cys, 100 μM Cd + 2500 μM DTT, 100 μM Cd + 300 μM BHA or 100 μM Cd + 2500 μM GSH for 6 h (Figure 4e), before performing luciferase imaging on the roots and quantifying the relative luciferase luminescence.

nrc1 and *cad2-1* enzyme activity experiments

The activity of γ-ECS was measured in protein extracts using HPLC fluorescence and thiol-derivatization with monobromobimane, using a modified version of the protocol described by Hell and Bergmann (1990). Rosette leaves from wild-type (Col-0), *nrc1* or *cad2-1* were ground in liquid nitrogen and 100-300 mg of ground tissue were mixed 1:1 with extraction buffer (100 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 8.0). Unless otherwise stated, all steps were carried out at 4°C and all buffers were saturated with nitrogen prior to being used to minimize the oxidation of thiols during protein extraction and enzymatic activity measurements. The protein extract was centrifuged for 10 min at 10 000 ***g***, and the supernatant was desalted using Sephadex G-25 previously equilibrated with extraction buffer. The enzyme activity assay (500 μl final volume) contained 100 mM Tris (pH 8.0), 50 mM $MgCl_2$, 20 mM glutamate, 5 mM ATP and an ATP regenerating system that consisted of 5 mM phosphoenolpyruvate, 1 mM DTT and 10 U ml^{-1} of pyruvate kinase. The protein extract was incubated for 10 min at 30°C, and γ-ECS activity was started by adding 1 mM cysteine. At defined time points, 50 μl of the reaction solution were taken and thiols were derivatized by adding 40 μl of monobromobimane (2 mM) and incubated for 20 min at 40°C. Derivatization was stopped by adding 10 μl of PCA30% v/v. Samples were mixed using a vortex and protein was precipitated by centrifugation (10 min at 10 000 ***g***). The supernatant was filtered using 0.45-μm Ultrafree-MC filters (Amicon; Millipore) before being analyzed by HPLC as described previously (Mendoza-Cozatl *et al*., 2008). The quantity of γ-EC synthesized over time was normalized to the protein content in the plant extract, quantified using the Bradford reagent (Sigma-Aldrich) and BSA as a protein standard.

nrc1 and *nrc2* complementation

All primers used for PCR amplification for cloning are listed in Table S2. For constitutive *γ-ECS* expression in the *nrc1* mutant, a *γ-ECS* genomic DNA fragment was amplified from Col-0 gDNA using the primers TJ199-F and TJ199-R (0-3294 bp). The amplified genomic *γ-ECS* DNA fragment, which excluded both the 5′ and 3′ untranslated regions (UTRs), was cloned into pENTR/D-TOPO® (Invitrogen), following the manufacturer's instructions. The *CaMV 35S*:γ-ECS-NOS construct was obtained by recombining the *γ-ECS* genomic sequence into a Gateway®-compatible pGreenII plasmid (Hellens *et al*., 2000) containing the $35S_{pro}$ and the *NOS* terminator ($35S_{pro}$:GW-NOS_{ter}), using LR Clonase II® (Invitrogen).

The pGREE-NII *35S:γ-ECS-NOS* construct was transformed using electroporation into *Agrobacterium tumefacians* strain GV3101.

For constitutive *GS* expression in the *nrc2* mutant, a *GS* genomic DNA fragment was amplified from Col-0 gDNA using the primers TJ198-R and TJ198-R (0-2702 bp). The amplified genomic *GS* DNA fragment, which excluded both the 5′ and 3′ UTRs, was cloned into pENTR/D-TOPO® (Invitrogen), following the manufacturer's instructions. The *CaMV 35S*:GS-NOS construct was obtained by recombining the *GS* genomic sequence into a Gateway®-compatible pGreenII plasmid (Hellens *et al*., 2000) containing the $35S_{pro}$ and the *NOS* terminator ($35S_{pro}$:GW-NOS_{ter}), using LR Clonase II® (Invitrogen). Note that our attempts to complement *nrc2* using a *35S*-driven *GS* cDNA were not successful, whereas the genomic DNA complimented the *nrc2* growth and yellowing phenotype (Figure S1) in six independent lines (all isolated transformants). We tested the complementation of the Cd-dependent root growth and thiol accumulation in three of these lines, as shown in the results section. The pGREENII *35S:GS-NOS* construct was transformed using electroporation into *Agrobacterium tumefacians* strain GV3101.

Arabidopsis thaliana was transformed using the floral-dip method (Clough and Bent, 1998) with the GV3101 strains described above. The helper plasmid, pSoup, was used for the pGreenII-carrying strains (Hellens *et al*., 2000). The pGREENII *35S:GS-NOS* construct was used to transform the *nrc2* mutant for complementation and Col-0, whereas the pGREENII *35S:γ-ECS-NOS* construct was used to transform the *nrc1* mutant for complementation. Hygromycin selection of transformants was performed in both the T_1 and T_2 generations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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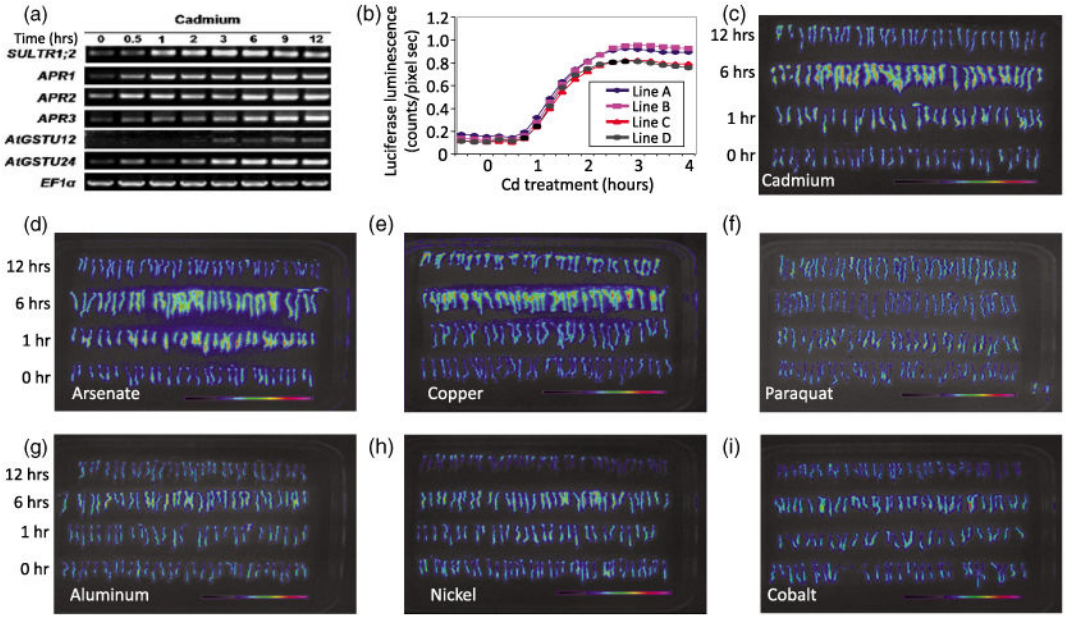

Figure 1.

Development of a cadmium-inducible reporter line. (a) Genes identified by microarray analysis as being induced following 200 μM cadmium (Cd) treatment for 12 h were verified by RT-PCR. Tissue was collected every 30 min and RNA was extracted as described in the Experimental Procedures. (b) Quantification of the luciferase activity of *pSULTR1;2:LUC* showing the dynamic response of four independent *pSULTR1;2:LUC* reporter lines over a 4-h time course. Measurements were taken in 15-min intervals.

(c–h) False color luminescence images after exposure to heavy metals, metalloids and reactive oxygen species (ROS)-generating compounds over a 12-h time course. Seedlings were exposed to 200 μM of the indicted substance and imaged as described in the Experimental Procedures. Specificity of the control reporter line response was determined by luminescence imaging after exposure to (c) cadmium, (d) arsenate, (e) copper, (f) paraquat, (g) aluminum, (h) nickel and (i) cobalt over a 12-h period.

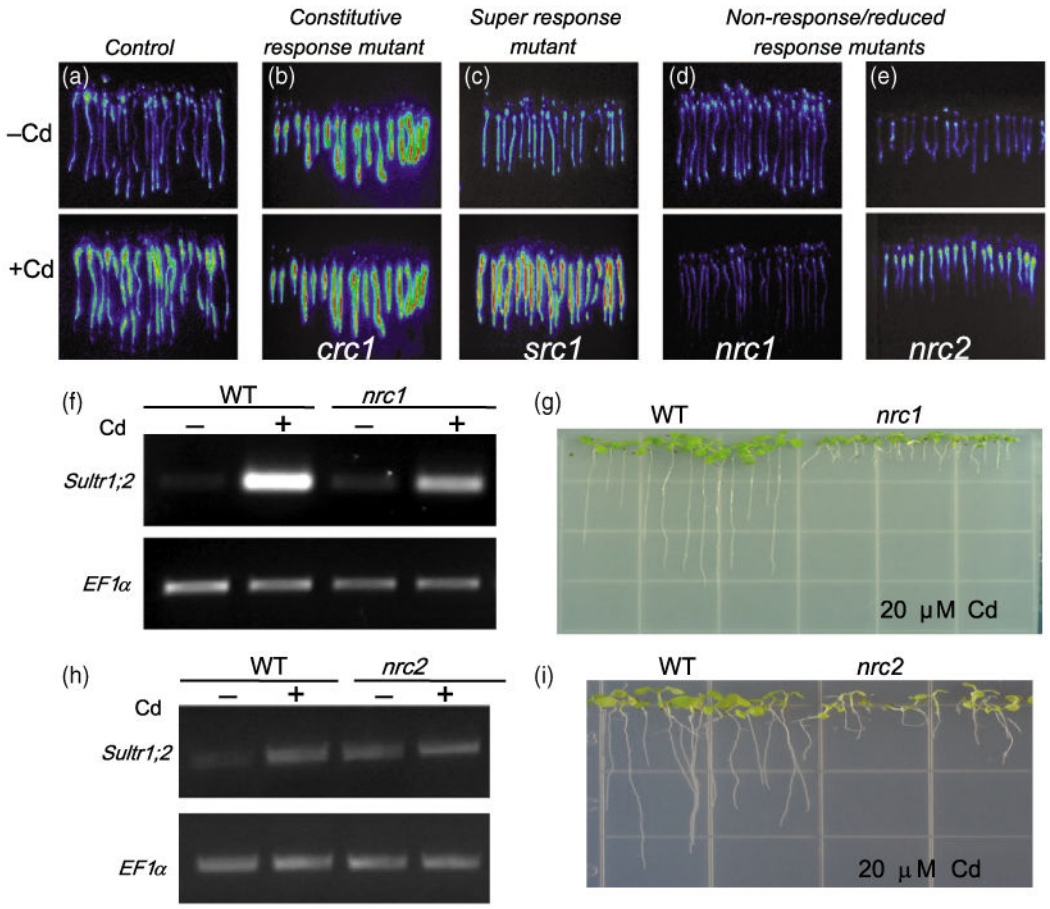

Figure 2.

Ethyl methanesulfonate (EMS)-mutagenized *pSULTR1;2:LUC* seeds were screened for altered cadmium-induced luciferase activity responses: (a) mutants were classified into three different groups based on luciferase response, compared with the control reporter line; (b) *constitutive response to cadmium* or CRC mutants (*crc1*); (c) *super response to cadmium* or SRC mutants (*src1*); (d) *non-response to cadmium* or NRC mutants (*nrc1*, d; *nrc2*, e).

(f) RT-PCR of the native *SULTR1;2* expression using 35 PCR cycles confirmed the NR reporter phenotype of *nrc1*.

(g) *nrc1* shows a short root phenotype compared with control when grown on 20 μM cadmium for 9 days.

(h) RT-PCR of the native *SULTR1;2* transcript using 30 PCR cycles showed a reduced induction in the *nrc2* mutant. (i) *nrc2* also has a short-root phenotype compared with the control when grown on 20 μM Cd for 14 days.

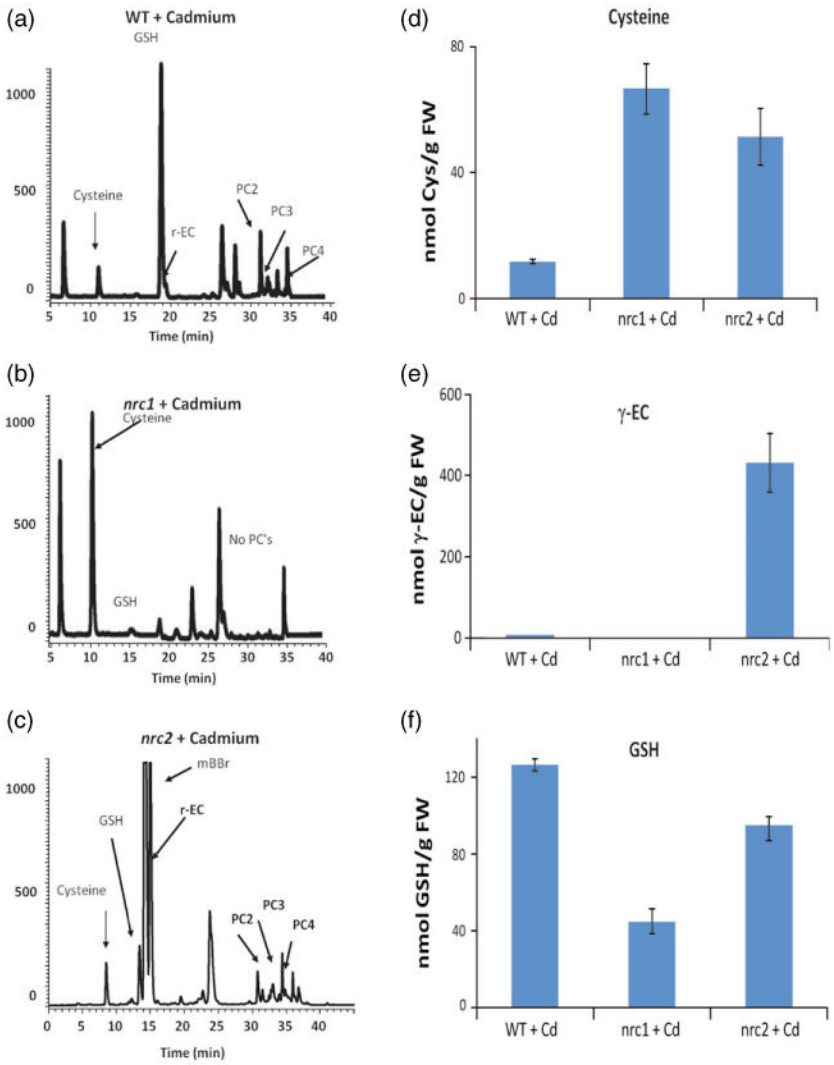

Figure 3.

Thiol profile of the *nrc1* and *nrc2* non-response mutants reveals enhanced cysteine levels in *nrc1* and enhanced γ-EC levelsin *nrc2*. Fluorescence HPLC-MS was used to identify and quantify thiols

(a) in control plants; (b) in the *nrc1* mutant; (c) in the *nrc2* mutant.

(d) Cysteine quantification using HPLC-MS revealed a five to sixfold accumulation of cysteine in both the *nrc1* and *nrc2* mutants relative to the control following treatment with cadmium.

(e) Fluorescence HPLC-MS also revealed a approximately 50-fold accumulation of γ-glutamylcysteine in *nrc2* relative to the control and a depletion of γ-EC in the *nrc1* mutant (approximately 25% of wild-type levels).

(f) Glutathione quantification using HPLC-MS revealed that both the *nrc1* and *nrc2* mutants are GSH deficient. All bar graphs show the mean of between three and six independent samples. Error bars represent the standard error of the mean (SEM).

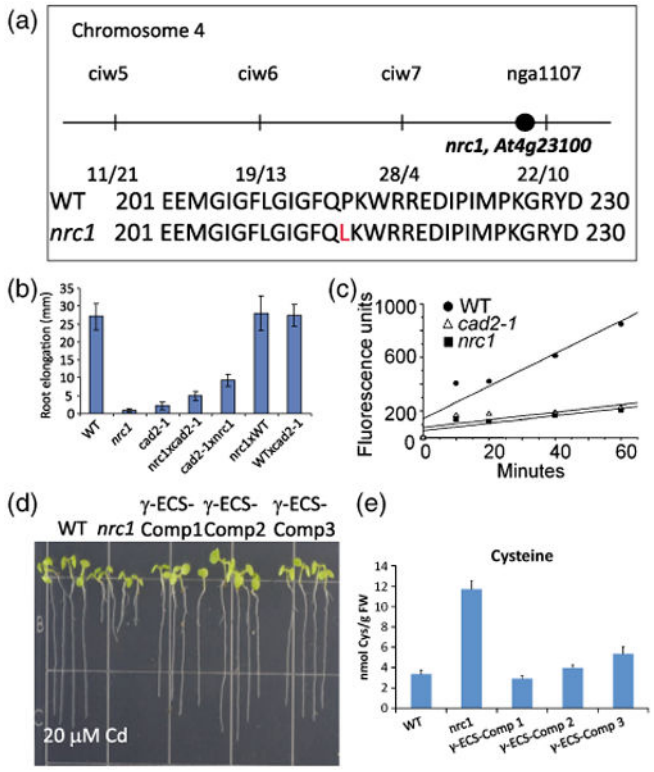

Figure 4.

Characterization of the non-response to cadmium mutant *nrc1*.

(a) Mapping of the *nrc1* mutant placed the mutation on chromosome 4 between the ciw7 and nga1107 markers. Candidate gene sequencing identified a point mutation in At4g23100, which causes a Pro → Leu (P214L) change in the γ-glutamylcysteine synthetase protein.

(b) Crosses between *nrc1* and *cad2-1* show the mutants are allelic. The short-root phenotype of the *nrc1* and *cad2-1* mutants grown on 20 µM cadmium (Cd) is not rescued in the *nrc1* × *cad2-1* or the *cad2-1* × *nrc1* F_1 cross, but crossing either mutant into WT restores root elongation in the F_1 generation.

(c) *nrc1* is deficient in γ-EC synthetase (γ-ECS) activity. An *in vitro* assay was performed to determine the activity of the γ-ECS protein from wild-type (WT), *nrc1* and *cad2-1* plants. γ-EC synthetase activity was measured by tracking γ-EC appearance using HPLC-MS in crude extracts (rosette leaves) obtained from wild-type Col-0 (full circles), *nrc1* (full squares) and *cad2-1* (empty triangles). Extracts obtained from WT plants showed a steady synthesis of γ-EC during the assay, whereas *nrc1* and *cad2-1* showed only marginal increases in γ-EC concentration during the assay.

(d) Expression of genomic γ-ECS in the *nrc1* mutant behind a constitutive promoter restores root elongation on Cd. Three independent T_2 transformants (Comp1–Comp3) were used for root elongation experiments on 20 µM Cd.

(e) Seven-day-old complementation lines (γ-ECS Comp1–Comp3) were also used for fluorescence HPLC-MS analyses following 48 h of 100 µM Cd treatment. Cysteine levels in the *nrc1* mutant are three to sixfold higher than in the WT (66.6 ± 8.22 nmol Cys per g fresh weight in *nrc1* compared with 11.8 ± 0.71 nmol Cys per g fresh weight in WT), whereas all three of the γ-ECS complemented (γ-ECS-Comp) lines show a WT cysteine accumulation.

All bar graphs show the mean of between three and six independent samples. Error bars represent the standard error of the mean (SEM).

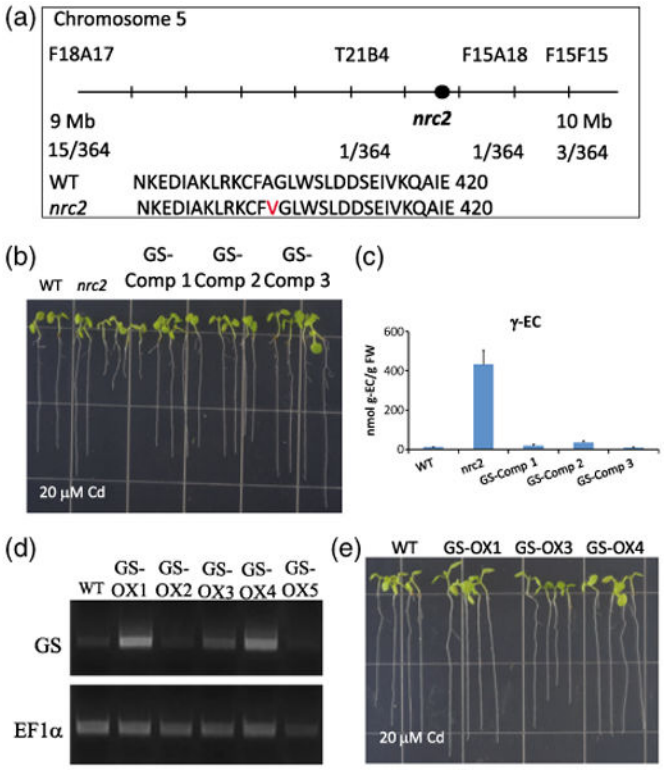

Figure 5.

Characterization of the *nrc2* non-response to the cadmium mutant as a first recessive viable allele in the *Arabidopsis* glutathione synthetase gene.

(a) Physical mapping of the *nrc2* mutant placed the mutation on chromosome 5, between the F18A17 and F15F15 markers. Candidate gene sequencing identified a point mutation in At5g23780, which causes an Ala → Val (A404V) change in the glutathione synthetase protein.

(b) Expression of genomic *GS* in the *nrc2* mutant behind a constitutive promoter restores root elongation on Cd. Three independent T_2 transfor-mants (GS Comp1–Comp3) were used for root elongation experiments on 20 μM Cd.

(c) Seven-day-old complementation lines (GS Comp1–Comp3) were also used for fluorescence HPLC-MS analyses following 48 h of 100 μM Cd treatment. Gamma-glutamylcysteine (γ-EC) levels in the *nrc2* mutant are approximately 50-fold higher than in the WT (431.7 ± 72.7 nmol per g fresh weight in *nrc2* compared with 8.57 ± 0.25 nmol per g fresh weight in WT), whereas all three of the *GS* Comp lines show a WT γ-EC accumulation.

(d) The same genomic *GS* construct was also used to transform WT Col-0 plants to produce GS overexpression lines (GS OX1-OX5). RT-PCR of the *GS* gene was performed on T_2 seedlings to identify lines expressing increased *GS* transcript levels compared with WT.

(e) T_2 *GS* overexpression lines (GS-OX1, GS-OX3 and GS-OX4) were then used for root elongation experiments to show that extopic *GS* expression does not increases tolerance to 20 μM Cd. All bar graphs show the mean values from between three and six independent samples. Error bars represent the standard error of the mean (SEM).

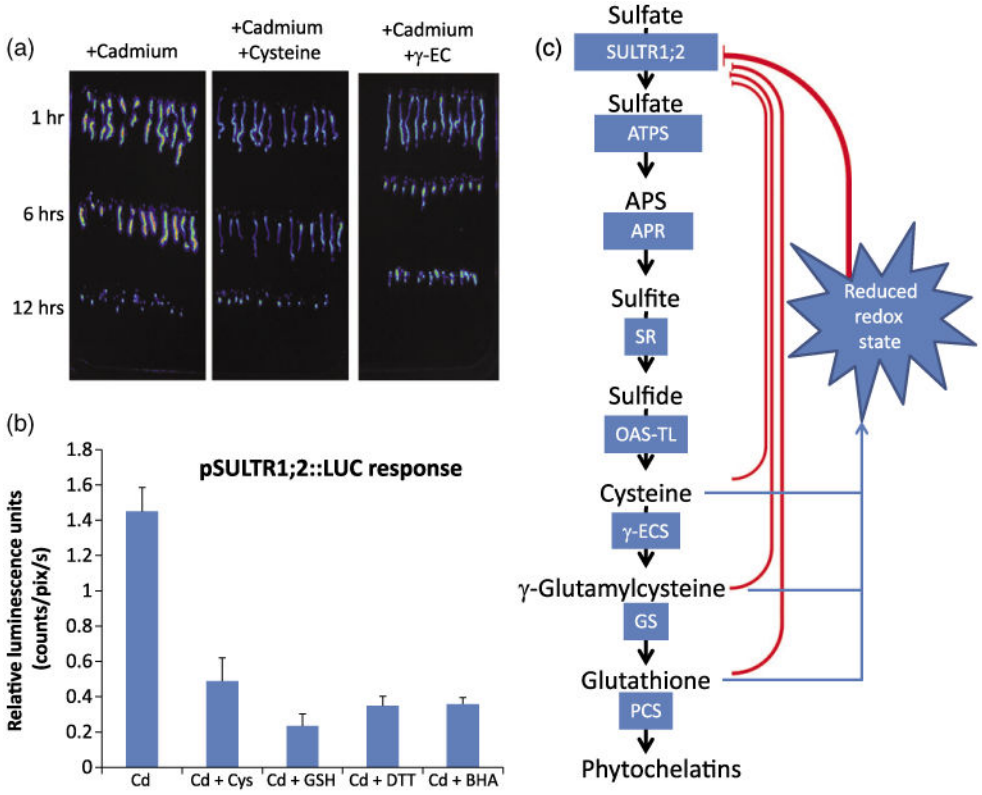

Figure 6.

Reducing compounds affect cadmium-dependent induction of the *pSULTR1;2:LUC* reporter.

(a) Addition of 1 mM cysteine or γ-EC to the growth media lowers the response of the control *pSULTR1;2:LUC* line to cadmium over a 12-h time course.

(b) Cadmium-dependent induction of the luciferase reporter gene is affected by reducing compounds. Exposure to 100 μM $CdCl_2$ induces the transcription of the *pSULTR1;2:LUC* reporter gene (Cd). The addition of the reducing compounds cysteine (Cd + 1 mM Cys) or glutathione (Cd + 1 mM GSH) to the growth media strongly represses the cadmium-induced luciferase activity. The non-physiological reducing agents DTT (Cd + 1 mM DTT) and BHA (Cd + 200 μM BHA) also repressed the induction of the reporter gene, suggesting that changes in the cellular redox state is a factor controlling the expression of the *SULTR1;2* gene. All images were quantified after 6 h of exposure to the specified compound.

(c) Schematic representation of putative signals involved in the regulation of the high-affinity sulfate transporter, *SULTR1;2*, during cadmium stress. Glutathione and cysteine are known to repress *SULTR1;2* under many conditions, including sulfur starvation and cadmium stress. Characterization of the *nrc1* and *nrc2* mutants suggests, however, that both GSH demand and changes in cellular redox are required for Cd-dependent transcriptional changes.