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Review Article

Cellular and signaling mechanisms supporting cadmium tolerance in salicylic acid treated seedlings

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Abstract

This review spotlights on recent indications that recognizes potential cellular mechanisms that may be involved in the tolerance of salicylic acid (SA)-treated seedlings to the presence of cadmium (Cd) in their environment. It appears probable that SA stimulates signaling systems implicated in plant defense-related actions against Cd-induced oxidative stress. These include mechanisms that reduce uptake of metals into the cytosol by extracellular chelation through extruded ligands and binding onto cell-wall constituents. Cellular chelation of metals in the cytosol by a range of ligands (peptides, phytochelatins (PCs)), or increased efflux from the cytosol out of the cell or into sequestering compartments are also key mechanisms improving tolerance. Free-radical scavenging capacities through the activity of antioxidant enzymes or production of peptides and PCs add another line of defense against the toxic effect of Cd. The SA signaling events can be attributed to the extracellular SA perception model in which reactions between SA and apoplastic proteins result in acute oxidative burst under Cd stress.

Keywords
Cadmium tolerance; salicylic acid; intracellular chelation; extracellular chelation; signal transduction

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Introduction

Exposure to cadmium (Cd) may occur naturally from metalliferous rocks, or from anthropogenic sources such as industrial pollution inputs, is with high level of acute toxicity for humans and animals (Irfan *et al.*, 2013; 2015). For plants, the degree of metal toxicity depends mainly on the concentration, way and duration of exposure as well as the age, genetics, and nutritional status of exposed seedlings (Djebali *et al.*, 2002). Furthermore, Cd bioavailability is a function of biotic factors such as mycorrhizal fungi and bacteria (Hall, 2002). In view of this, salicylic acid (SA) is one of the key hormonal factors determining the providence of plants grown on contaminated sites, which is naturally found in plants and shown to be involved in the alleviation of Cd toxicity (Guo *et al.*, 2007, 2013; Li *et al.*, 2014; Belkadhi et *al.*, 2015 a, b).

The local SA signaling actions can be recognized to (i) the extracellular SA action model and/or (ii) intracellular SA perception model (Kawano and Bouteau, 2013). On the other hand, the long distance SA action could be credited to three singular approaches, namely, iii) local increase in SA followed by transportation of SA, (iv) systemic propagation of SA developed movable signals without direct movement of SA, and (v) synergistic

proliferation of both SA and movable signals through the tissues and phloem. This includes the alternately repeated secondary signal proliferation and synthesis and/or liberation of SA finally contributing to the systemic spread of SA-derived signals.

Previous studies have recapitulated the available data on amelioration of Cd toxicity by exogenous SA applications (Metwally *et al.,* 2003; Choudhury and Panda, 2004; Guo *et al.,* 2007; Li *et al.,* 2014; Belkadhi *et al.,* 2015 a, b; Xu *et al.,* 2015), and this will be as well considered here. In addition, we will focus on the mechanisms involved in improving physiological, cellular, and molecular responses to Cd stress (Atkinson and Urwin, 2012). In fact, various mechanisms potentially involved in Cd tolerance have been characterized in SA-treated seedlings and can be described as extracellular (binding to the cell-wall) or intracellular (binding to peptides and transport into intracellular compartments) detoxification systems. Extracellular mechanisms are mainly implied in avoidance of Cd entry, whereas intracellular systems aim to reduce metal burden in the cytosol. Supplementary antioxidative detoxification mechanisms, which allow the alleviation of reactive-oxygen species (ROS) toxicity, indirectly initiated by Cd, may be part of improvement of tolerance mechanisms in SA-treated seedlings.

Role of SA in Cd chelation and compartmentalization

SA is generally considered as an important agent of improving plant resistance to Cd stress probably through its stimulating action of hydrogen peroxide (H_2O_2) release from cells (Belkadhi *et al.,* 2014). In particular, SA can form chelate compounds with Cd ions (Perrin, 1958). The specific mechanism that involves SA in Cd chelation is not yet explored (Belkadhi *et al.,* 2012). However, Ooto (1975) hypothesized the belief that free o-hydroxyl group on benzoic acid confers metal chelating characteristic to this molecule. A moderate resistance to heavy metals can be realized by selective Cd exclusion, moderated uptake, or energetic efflux from the roots, especially by mechanisms leading to lower cytoplasmic Cd contents (Hall, 2002). Nevertheless, Cd tissue contents were unaffected, both at the whole-plant and organ level, in mesophyll cells and vacuoplasts, ruling out the involvement of differential transport of Cd between plant organs and across the plasma membrane as a physiological reason for the beneficial effect of SA (Metwally *et al.,* 2003).

Members of the ABC transporter family are identified to be implicated in vacuolar sequestration of toxic metals (Kim *et al.,* 2007; Kang *et al.,* 2011). Transcript levels of some Arabidopsis ABC transporters are modified in response to SA (Kang *et al.,* 2011). Such transporters may improve vacuolar sequestration of Cd in the SA-treated seedlings (Metwally *et al.,* 2003). Metwally *et al.* (2003) also showed that SA content was increased in Cd-stressed seedlings and the possible formation of stable SA-Cd complexes that lowered Cd toxicity after SA pretreatment was expected. Likewise, Cd-SA complex formation in the hydroponics solution is an improbable reason for the favorable effect of SA since the exposure to Cd started 3 d after the SA pretreatment in the presoaking experiment and 24 h afterward in the pretreatment experiment (Belkadhi *et al.,* 2013; 2014). However, complex formation might have played a role in the short-term experiments with leaf slices (Metwally *et al.,* 2003).

SA and complexation of Cd by peptides

Despite intracellular chelation capacities of tolerant plant species to heavy metals, certain amount of toxic free Cd ions may be concentrated in the cytosol. Pre-treatment with SA has been shown to improve various mechanisms to cope with the presence of free Cd ions inside cells. These include molecular and biochemical modifications of root anatomy, the binding of Cd ions to the cell wall, the transportation of Cd complexes to the vacuoles for storage, the production of fewer toxic compounds by ways of methylation, and the excretion or secretion of Cd ions (Djebali *et al.,* 2002; 2005). The most frequent compounds involved in Cd tolerance are metal-binding peptides and phytochelatins (PCs). The former are cysteine-rich, found mostly in plants, capable of neutralizing toxic Cd ions and contain glutamic acid in a δ-bond, allowing them to form thiolate bonds with Cd ions (Rao *et al.,* 2006) and thus protect plant cells from damage. On the other hand, PCs are important not only due to their metal-binding capacity, but also because they transport the bound metal ions from the cytoplasm into the vacuoles, where they can be stored as non-toxic form, bound to organic acid ligands (Brunetti *et al.,* 2011; 2015).

One of the earliest studies to report on the protective effect of SA against Cd exposure of seedlings dealt with the role of PCs in the SA-mediated protection against Cd toxicity. Grown hydroponically in solution containing 0, 0.01, 0.015, or 0.025 mM Cd, Szalai *et al.* (2013) reported that presoaking seeds of *Zea mays* in 0.5 mM SA before exposure to Cd reduced the level of heavy metal injury and has an effect on the composition of individual PCs; however this protection was not directly connected with the altered regulation of PCs. Lately, Freeman *et al.* (2005) have recommended a model for the involvement of SA in the Ni tolerance of *Thlaspi* hyperaccumulators. Indeed, SA activates Ser-acetyltransferase (EC 2.3.1.30) (Freeman *et al.,* 2004) post-translationally leading to increase in GSH synthesis and activation of GSH reductase (Knörzer *et al.,* 1996) to preserve an improved pool of reduced GSH. Furthermore,

SA potentially blocks PC synthase activity (Pál *et al.,* 2002), slowing down PC biosynthesis in response to Ni and shielding GSH to operate as an antioxidant (Freeman *et al.,* 2005), thus preventing Ni-induced oxidative stress in *Thlaspi* hyperaccumulators. In an earlier experiment, it was also proved that the GSH-mediated Ni tolerance mechanism observed in Ni-hyperaccumulating Thlaspi species is signaled by the constitutively elevated levels of SA. It was also observed that both biochemical and genetic manipulations that increase SA in *Arabidopsis thaliana* seedlings imitate the GSH-related phenotypes of the hyperaccumulating *Thlaspi*, and that these biochemical changes in the non-accumulator plants are associated with increased GSH-mediated Ni resistance (Freeman *et al.,* 2004). Such results suggested that SA may perhaps be one of the regulators implicated in monitoring certain key biochemical differences between Ni/Zn hyper-accumulators and non-accumulator plant species. Purposely, tolerance of *Thlaspi* hyperaccumulators to Cd, could be affected by a similar mechanism (Freeman *et al.,* 2005). In any case, the role of SA and GSH to prevent oxidative stress in different plant species showed different levels of Cd tolerance that still needs to be elucidated.

Influence of SA on transport mechanisms involved in Cd tolerance

There has been little recent work on the transport of heavy metals in SA-treated seedlings. Apoplastic proteins may be involved in Cd tolerance either by extruding the toxic metal ions from the cytosol out of the cell or by allowing metal sequestration into intracellular compartments (Williams *et al.,* 2000; Hall, 2002). Using radiotracer flux analyses, the significant accumulation of Cd found in the higher plants, involves the accumulation of Cd-conjugated peptides or Cd-conjugated PCs in the vacuole. This process appears to be mediated by the ATP-binding cassette transporters located at the tonoplast (Ortiz *et al.,* 1995). In fact, ATP-binding cassette (ABC) transporters are known to be involved in vacuolar sequestration of toxic metals (Clemens, 2006). A new ABC transporter has been identified as a SA-induced gene from *Glycine max* (Eichhorn *et al.,* 2006). This transporter might facilitate vacuolar sequestration of Cd in the SA-treated plants. Belkadhi *et al.* (2015b) assumed that the beneficial effects of exogenous SA pre-treatment can be related to modification of Cd compartmentalization in vacuoles. As, this molecule can form a complex with Cd that may provide tolerance to Cd stress (Moussa and EL-Gamal, 2010). Otherwise, it is well known that Cd entry via essential element transporters is the basis process of its uptake by root cells (Hall and Williams, 2003). This absorption and transport of Cd happens because of the similarity in the electron structure between the heavy metal and essential cations (Cosio *et al.,* 2004).

Additionally, Singh *et al.* (2015) reported that exogenous application of SA reverted the growth, and oxidative stress caused by arsenic V (As V) and significantly decreased As translocation to the shoots. Then, the level of iron (Fe) in root and shoot was completely interrelated with the transcript level of transporters responsible for the accumulation of Fe, OsNRAMP5, and OsFRDL1, in both plant organs. The authors concluded that these observations might be due to SA-induced down regulation of As transporters from root to shoot. In a previous study OsLsi2, transporter responsible for AsIII transport from root to shoot in rice (Ma et al., 2008), has been found to be down regulated at mRNA level. Since AsIII is the principal intracellular form (Ma *et al.,* 2008; Mishra *et al.,* 2013) and also probably the main As groups translocated to the shoots. Consequently, down regulation of OsLsi2 would negatively affect the As accumulation. Furthermore, Srivastava *et al.* (2014) explained that down regulation of OsLsi2 was owed to minor As concentration in rice shoots in response to thiourea supplementation with As. OsLsi1 is primarily responsible for AsIII transport to root was not related to As uptake in roots (Singh *et al.,* 2015; Armendariz *et al.,* 2016). Otherwise, SA has been revealed to activate ABC transporters in *Glycine max* (Eichhorn *et al.,* 2006). These transporters are responsible for the vacuolar sequestration of As(III)-PC forms (Song *et al.,* 2010). Therefore, it may be probable that the lesser As transported to the shoot was due to the fact that most of the accumulated As in SA treated rice seedlings were sequestered in root vacuoles as As(III)-PC form. Further, SA pre-treatment has been described to improve PCs synthesis in maize roots (Szalai *et al.,* 2013).

Since SA mediated resistance against Cd stress has reported in previous studies (Metwally *et al.,* 2003; Shi and Zhu, 2008), some reductions in the level of accumulation were observed (Belkadhi *et al.,* 2010). For this purpose, less concentration of metalloid in shoot may also have an effect on its level in grain which would have great implications with respect to human toxicity through food chain Cd contamination. Besides, the beneficial effect of SA on Cd bioaccumulation could be attributed to the augmentation of the uptake of cations (Ca, Mg and Fe) in Cd-treated plants (Belkhadi *et al.,* 2010). Within soybean cells, Dean and Mills (2004) reported that SA is converted primarily, in the cytoplasm, to SA 2-O-beta-d-glucose (SAG) and then accumulates, wholly, in the vacuole.

However, the mechanism involved in the vacuolar transport of SAG has not been investigated. By measuring the [(14)C]SAG uptake into tonoplast vesicles isolated from etiolated soybean hypocotyls, the authors characterized the vacuolar transport of SAG. They found that the uptake of SAG was stimulated about 6-fold when Mg-ATP was included in the assay media. In contrast, after adding Mg-ATP, the uptake of SA was only stimulated by 1.25-fold and was 2.2-fold

less than the uptake of SAG providing an indication that the vacuolar uptake of SA was promoted by glucosylation. Based on the characteristics of SAG uptake into soybean tonoplast vesicles, authors concluded that this uptake occurred through an ABC transporter-type mechanism. Nevertheless, this uptake mechanism in vacuoles is not common since the uptake of SAG by *Beta vulgaris* L. tonoplast vesicles required an $H^{(+)}$-antiport mechanism (Dean and Mills, 2004).

SA induced oxidative signaling events leading to Cd stress adaptation

Systemic signals are observed in separate plant tissues and initiate systemic stress resistance through priming or else stimulation of defense reactions (Aranega-Bou *et al.,* 2014). Recently, the knowledge on such long-distance signaling has been documented through the studies on systemic acquired resistance (SAR), systemic acquired acclimation (SAA), and systemic wound response (SWR) (Aranega-Bou *et al.,* 2014). According to previous studies, phloem is the likely path for systemic transmission or movement of signals associated with those three SA-induced responses. Correspondingly to SAR induction following the challenges by abiotic environmental conditions can be the elicitors for SA-centered signaling cascade finally leading to SAA in the challenged plants.

Reactive oxygen species (ROS) are often unavoidably generated by living organisms as by-products from normal metabolic reactions including respiration and photosynthetic processes, and fatty acid metabolism (Moller, 2001; Baker *et al.,* 2006; Noctor *et al.,* 2012). A widespread property of all forms of ROS is that they can cause oxidative damage to cellular components such as proteins, DNA, and membranes (Moller *et al.,* 2007). The specificity of the biological response of living plant cells to ROS counted on the chemical characteristics of ROS, strength of the signal, sites of production, and growth stages (Del Rio *et al.,* 2002). Consequently, apart from their harmful action, generation of ROS could be potentially advantageous to living cells depending on the environmental conditions (Apel and Hirt, 2004). Induced production of ROS like hydrogen peroxide (H_2O_2), superoxide anion radicals ($O_2^{\bullet}$) and hydroxyl radicals ($HO^{\bullet}$), at the cell walls via the apoplast pathway (Bolwell *et al.,* 2002) recognized as the “oxidative burst”, is one of the earliest events detectable during Cd stress (Olmos *et al.,* 2003; Sharma *et al.,* 2012).

Exposures to Cd stress reportedly increase the intra- and intercellular levels of H_2O_2 by modulating the finely highly structured ROS detoxification networks, composed of ROS-producing enzymes, antioxidant enzymes, and biosynthetic pathways for low molecular weight antioxidants, all responsible for maintaining the homeostasis of ROS levels (Bolwell *et al.,* 2002; Del Rı´o *et al.,* 2002; Kawano *et al.,* 2003; Kotchoni and Gachomo, 2006; Yoshioka *et al.,* 2008). Olmos *et al.* (2003) demonstrated a rapid generation of H_2O_2 by Cd^{2+}-treated plant cells was in cultured tobacco (*Nicotiana tabacum* L.) BY-2 cells. By using cytochemical methods, they had located the starting point for the generation of H_2O_2 at the cell plasma membrane. Exogenous application of diphenyleneiodonium (DPI) and imidazol, both inhibitors of the neutrophil NADPH oxidase, prohibited the production and propagation of H_2O_2 induced by Cd^{2+}. These data suggested the involvement of an NADPH oxidase-like enzyme leading to H_2O_2 generation through $O_2^{\bullet-}$ dismutation by superoxide dismutase enzymes. Then, to investigate the implication of Ca^{2+} channels in a Cd^{2+}-induced oxidative burst, Olmos and collaborators (2003) had used different inhibitors of Ca^{2+} channels. Results showed that only La^{3+} totally inhibited the generation of H_2O_2 induced by Cd ions. Nevertheless, the inhibitors of Ca^{2+} channels (verapamil and nifedipine) were not effective. Furthermore, based on the results obtained with fluphenazine and N-(-6-amino-hexyl)-5-chloro-1- naphthalenesulphonamide (W-7) and staurosporine, two types of calmodulin antagonists, an inhibitor of protein kinases, calmodulin or a Ca^{2+}- dependent protein kinase was also implicated in the signal transduction sequence. However, neomycin, an inhibitor of the phosphoinositide cycle, did not inhibit the formation of H_2O_2 induced by Cd^{2+}, signifying essentially an induction of the oxidative burst mediated by calmodulin and/or calmodulin-dependent proteins.

A series of previous and recent works (Olmos *et al.,* 2003; Sharma *et al.,* 2012) demonstrated for the first time that ROS generation occurs in plants after the exposure to the heavy metal and that the members of ROS possibly function as the chemical signals necessary for induction of hypersensitive response (HR) which is believed to be mediated by endogenous SA (Coll *et al.,* 2011; De Pinto *et al.,* 2012). This allows ROS to serve as signaling molecules in plants in order to regulate the metabolism and the cell signal transduction pathways activated in response to the environmental stress (Gechev *et al.,* 2006; Mittler *et al.,* 2011).

Collected evidences established that hormonal signaling pathways inducing development of SAR are regulated under restricted ROS generation as observed for SA, jasmonic acid (JA), and abscisic acid (ABA) (Kawano, 2003; Mori and Schroeder, 2004; Al-Hakimi, 2007; Gaupels *et al.,* 2011; Maksymiec, 2011; Wen *et al.,* 2011; Kumar *et al.,* 2012; Masood *et al.,* 2012). Similar hormonal regulations mediated by ROS might be involved in the cross talk between abiotic stress signaling pathways (Stroher and Dietz, 2006). Although many constituents of oxidative signaling network pathways have been identified in recent times, the mechanisms for orchestrated control of the

expanded ROS generation processes at diverse cellular sites through the improvements of ROS feedback control to congregate the physiological necessities such as plant growth and development and adaptation to biotic and abiotic stress are currently actively studied (Coll *et al.,* 2011).

On the other hand, many studies reported that the Cd stress signal might also be transmitted by SA molecules (Rodríguez-Serrano *et al.,* 2006; Yakimova *et al.,* 2006; Krantev *et al.,* 2008; Stroiński *et al.,* 2013). Moreover, accumulation of SA under Cd stress has been noted in pea, maize and *Arabidopsis* plants (Rodríguez-Serrano *et al.,* 2006; Zawoznik *et al.,* 2007). Additionally, Poovaiah *et al.* (2013) revealed that expression of genes controlling the defense responses after the accumulation of SA seemed to be regulated by Ca^{2+}/calmodulin-mediated signaling. In fact, calmodulin is a signal molecule which is crucially involved in the signaling cascades that generate the Cd-induced oxidative burst (Garnier *et al.,* 2006). Besides, as a member of a family of crucial leucine zipper (bZIP) transcription factors for which competitive binding to calmodulin has been shown to be implicated in regulating the levels of expression of different genes (Neuhaus *et al.,* 1994).

Szymanski et al., 1996 showed that TGA3 (a member of the family of transcription factors TAGs) binding to calmodulin, enhances its interaction with the target promoter. Furthermore, TGA3 interrelates with NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1), one of the most important transcription cofactor involved in SA signal perception and the expression of a large range of genes controlling the defense responses; affording a possible option to regulate the output of defensive reactions (Fu and Dong, 2013). As a final point, it is very interesting to investigate the role of Ca^{2+}/ calmodulin in SA-treated seedlings after the exposure to Cd.

Conclusion

This review has assessed current researches that discovered potential cellular and signaling mechanisms that may be involved in the tolerance of SA-treated plants to the presence of Cd in their environment. These include improving mechanisms that decrease uptake into the cytosol by intracellular chelation of metal by means of ligands (peptides, PCs), or efflux from the cytosol into sequestering compartments or binding onto cell-wall components. Specific features such as the efficiency of PC synthesis have also been described. However, most of the molecular mechanisms prolong to be revealed, among which transport mechanisms are of great interest. Moreover, observations with a particular mode of application of SA in plants exposed to Cd must be generalized with caution. It appears for example that the foremost mechanism implicated in Cd detoxification in SA-treated plants consists on enhancing its compartmentation within the vacuole as sulphur-rich complexes, whereas Cd tolerance achievement could be primarily because of a reduced Cd accumulation within shoot and root cells. Away from these mechanisms, certaintly is the setback of understanding tolerance in SA-treated plants, and this brings in supplementary level of complexity that is beyond the scope of this review. SA cellular signaling mechanisms are likely linked through the import and export of SA by the cells. Since the low-affinity SA efflux carrier required for excretion of highly accumulated SA is reportedly induced by extracellular SA-induced oxidative burst and calcium signaling mechanism.

Competing Interests

The authors declare that they have no competing interests.

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