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Short communication

Profiling of proteasome activity in *Alyssum* species on serpentine soils in Turkey reveals possible insight into nickel tolerance and accumulation

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ABSTRACT

In crops and most plants, nickel induces oxidative stress resulting in oxidized and misfolded proteins. Proteasomes maintain cellular homeostasis during stress by removing these damaged proteins. Although mild stress tolerance is mediated by proteasomal proteolysis of misfolded and oxidized proteins, previous studies have observed that severe nickel stress decreases proteasome activity in nickel-sensitive plants. Whether or not proteasome function is impaired in nickel-tolerant plants is not know. Therefore, we tested the hypothesis that proteasome activity is elevated in nickel-tolerant *Alyssum* species capable of accumulating nickel to unusually high levels. Our field studies examined *Alyssum sibiricum* and *Alyssum caricum*, a moderate nickel accumulator and hyper-accumulator respectively, growing on their native serpentine soil in Turkey. *A. sibiricum* had higher proteasome activity on serpentine soil compared to non-serpentine soil; these plants also had elevated levels of nickel accumulation and higher proteasome activity compared to other low accumulating plants in the genus *Festuca* or *Astragalus*. In *A. caricum*, proteasome activity was very weakly correlated with nickel soil bioavailability or accumulation in leaf tissue, suggesting that proteasome function was not impaired in plants that accumulated the highest concentration of nickel. We discuss if maintained proteasome activity might underpin nickel tolerance and the unique ecophysiology of nickel hyper-accumulation in plants.

1. Introduction

One factor that limits global plant productivity is an abundance of heavy metals in soils. Accumulation of metals in plants is associated with oxidative stress, and is therefore toxic at higher levels to most plants (Apel and Hirt, 2004). Intriguingly, some rare plants have evolved mechanisms to tolerate and accumulate metals in their tissue. These unique plants are appropriately termed hyper-accumulators because they can accumulate metals to concentrations that exceed 1000-fold compared to most plants without showing adverse effects (Reeves et al., 2001; Rascio and Navari-Izzo, 2011). The elemental defense hypothesis predicts that these plants likely evolved the ability to hyper-accumulate metals and metalloids to prevent herbivory (Boyd, 2004; Cappa and Pilon-Smits, 2014).

Although plants can hyper-accumulate a variety of metals and metalloids, most are nickel (Ni) hyper-accumulators whose Ni concentrations are above 0.1% of their biomass (1 ppt). There are over 350 Ni hyper-accumulating species, most of which are primarily localized in the Mediterranean region and belong to the genus *Alyssum* (Krämer, 2010). In fact, the first metal hyper-accumulating plants were first discovered nearly 70 years ago when it was reported that *Alyssum bertolonii* accumulated 1% nickel (Minguzzi and Vergnano, 1948).

Although previous research has revealed key insight into Ni hyper-accumulating plants, they remain a botanical enigma, *i.e.* how do they preferentially accumulate Ni to levels that would induce stress in most plants (Seregin and Kozhevnikova, 2006)? Pioneering research by Krämer et al. (1996) identified the first molecular mechanism implicated in Ni hyper-accumulation by reporting that free histidine in *Alyssum* can bind to and form a complex with Ni. Since this discovery, other organic molecules have been reported to chelate Ni, including malate and citrate (Montargès-Pelletier, 2008; Agrawal et al., 2012). Evidence indicates that Ni-organic acid complexes are sequestered in the vacuole (Krämer et al., 2000). Ni chelation is an important tolerance mechanism in *Alyssum* hyper-accumulators, because it prevents Ni

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from (*i*) replacing metal cofactors in essential metalloenzymes (Ghasemi et al., 2009) and (*ii*) redox cycling with thiols leading to free radicals (Stohs and Bagchi, 1995) as observed in corn (Baccouch et al., 1998) and rice (Maheshwari and Dubey, 2009). To thwart the prooxidant activity of Ni, plants that hyper-accumulate Ni also benefit from increased glutathione (Freeman et al., 2004) levels and catalase activity (Boominathan and Doran, 2002).

Ni-induced accumulation of reactive oxygen species damages lipids and DNA, and promotes the misfolding and oxidation of proteins (Pena et al., 2008). These impaired proteins would lead to cytotoxic protein aggregates if they are not removed by the proteasome (Smalle and Vierstra, 2004). In eukaryotes, proteasomes maintain cellular homeostasis by tagging short-lived regulatory proteins with ubiquitin prior to their delivery to the proteasome (Coux et al., 1996). Proteolysis of ubiquitinated proteins occurs inside the catalytic core of the proteasome, which is bound by either 1 or 2 ATP-dependent regulatory lids. In contrast to regulatory proteins, oxidized proteins are removed by the proteasome independent of ubiquitin or attached lids (Davies, 2001).

The proteasome is associated with improved stress tolerance in plants confronting environmental factors that induce protein damage, *e.g.* heat, metals, and metalloids (Lyzenga and Stone, 2012). For example, the proteasome prevents toxicity in the selenium hyper-accumulator *Stanleya pinnata* by removing malformed selenoproteins that contain a selenocysteine to cysteine substitution (Sabbagh and Van Hoewyk, 2012). Proteasome activity is also elevated in *Chlamydomonas* (Vallentine et al., 2014) treated with selenite for 3 h; however this response was time and dose-dependent, as proteasome activity decreased during severe oxidative stress (Shang and Taylor, 2011). Prolonged salt (Wang et al., 2011) and heavy metal treatment also decrease proteasome activity and/or levels of ubiquitinated proteins (Pena et al., 2008; Karmous et al., 2014; Lee and Hwang, 2015). For example, when sunflower plants were treated with ten different metal(oid)s, decreased proteasome activity was most pronounced in Ni-treated plants (Pena et al., 2008), suggesting that excessive Ni directly disrupts the proteasome. *In vitro* experiments substantiate this possibility. Proteasomes isolated from both sunflower (Pena et al., 2008) and human cells (Frezza et al., 2009) exhibit decreased activity when incubated with Ni. In fact, it has been proposed that heavy metal toxicity might directly stem from its deleterious effects on proteasomes (Tomco et al., 2014). If Ni impairs the proteasome, this raises the question of whether or not maintenance of the proteasome during metal accumulation or severe stress bestows improved stress tolerance.

The objective of this study is to determine if elevated proteasome activity is associated with Ni accumulation in the *Alyssum* genus. To meet this challenge, we performed both lab and field studies in Turkey, which is well-known for its abundance of Ni hyper-accumulating plants that are endemic to serpentine soils rich in Ni (Reeves et al., 2001; Adıgüzel and Reeves, 2012; Altinözlü et al., 2012; Turgay et al., 2012). To our knowledge, this is the first time that proteasome activity has been reported in any hyper-accumulating plant.

2. Methods

2.1. Site selection

Sites selected to collect leaf tissue from *Alyssum sibiricum* and *Alyssum caricum* were previously identified (Reeves et al., 2001; Altinözlü et al. (2012); Adıgüzel and Reeves (2012). Ankara Beynam Forest in central Anatolia was previously reported to contain *A. sibiricum* that moderately accumulated Ni (< 1 ppt), whereas Mugla province near the Aegean Sea supported *A. caricum* that can hyper-accumulate Ni to levels greater than 1 ppt (Reeves et al., 2001) We visited these sites in July 2014 to confirm the presence of *A. sibiricum* and *A. caricum* on serpentine soils. Based on our preliminary work, this study selected sites in both Ankara Beynam Forest and Mugla (Fig. 1). In April 2016, we collected leaf and soil samples from these localities. In addition to the above-mentioned *Alyssum* species, we also collected *Festuca valesiaca* and *Astragalus* sp at the Ankara Beynam Forest site; both of these plants are not known to accumulate nickel and are considered nickel sensitive plants. Plants were identified in the field. Leaf tissue for ICP analysis was collected in paper bags; leaf samples for proteasome assay were immediately placed on dry ice and transported back to Ankara University for analysis.

2.2. Plant and soil Ni analysis

Ni concentration was measured as previously described by our group (Turgay et al., 2012). Briefly, leaf samples from *A. sibiricum* and *A. caricum* were washed with deionized water to remove residual nickel, dried at 65 °C for 3 days, and then pulverized before passing through a 0.2-mm sieve. Plant samples were heated in a muffle furnace at 500 °C for 6 h. After digestion in 5 mL of 2 M HNO_3, the samples were diluted to 25 mL with reverse-osmosis water. Extracts were filtered and stored in plastic vials until analyzed. Total Ni concentration in leaf tissue was determined on a Perkin Elmer 2100DV inductively coupled plasma optical emission spectrometer (ICP-OES) after diethylenetriaminepentaacetic acid (DTPA) extraction (Kalra, 1998; Baker and Amacher, 1982).

2.3. Proteasome activity assays

To determine proteasome activity, leaf tissue was ground in liquid nitrogen, as described previously (Dimkovikj and Van Hoewyk, 2014). Briefly, non-denatured proteins were extracted in cold proteasome extract buffer (50 mM potassium–phosphate buffer, pH 7.4, 5% glycerol, 10 mM ATP, and 5 mM beta-mercaptoethanol), and the extract was collected after centrifugation at 15,000 RPM. Protein concentration was determined using the Bradford assay (Bradford, 1976). Chymotrypsin activity of the proteasome was determined fluorometrically (Ex_{360}/Em_{410}) in a reaction containing 5 µl of protein extract and 95 µl of reaction buffer (50 mM potassium-phosphate buffer, 2 mM $MgCl_2$, 1 mM ATP, 5 mM β-mercaptoethanol, 50 µM of the fluorogenic peptide Suc-LLVY-AMC).

With or without 10 µM of the proteasome inhibitor MG132 dissolved in 0.1% dimethyl sulfoxide (DMSO). Activity was determined after 30 min as the fluorescence per microgram of protein in reactions, and is the difference in reactions with or without MG132 to account for non-proteasomal proteolysis. Proteasome activity is reported as relative fluorescent units (RFU) or the percentage relative to plants growing without Ni.

3. Results

3.1. A. sibiricum has higher proteasome activity than other species on serpentine soil

Initial experiments sought to determine if *A. sibiricum* plants had elevated proteasome activity compared to other species growing on serpentine soil in Turkey. To meet this objective, we utilized nearby field sites in Ankara Beynam Forest in Ankara, Turkey; this area was previously identified as sites containing populations of *A. sibiricum* (Reeves et al., 2001; Turgay et al., 2012). *Festuca valesiaca* and *Astragalus sp.* were selected as low Ni accumulating plants; we were unable to identify *Astragalus* plants at the species level. Populations of plants from the genus *Festuca valesiaca*, *Astragalus sp*, and *Alyssum* sibiricum were collected on sites containing serpentine soil, and individual plants were analyzed for their nickel content and proteasome activity. As expected, Festuca *valesiaca* and *Astragalus sp.* had lower concentrations of nickel content compared to *A. sibiricum* (Fig. 2A). Ni hyper-accumulation was not observed in any *A. sibiricum* plants collected, because the concentration of Ni was below 0.1% threshold to be considered a hyper-accumulator (Krämer, 2010); this result was also