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Phytochelatins govern zinc/copper homeostasis and cadmium detoxification in *Cuscuta campestris* parasitizing *Daucus carota*

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ABSTRACT

Cuscuta sp., known with the common name of "dodder", is an obligate parasite capable of invading stems and leaves of a wide range of host plants. Dodder stem usually coils counterclockwise around the host and, within a few days, develops haustorial structures at each point of contact. As soon as dodder haustoria reach host vascular bundles, they start tapping water, photosynthates and minerals. Metal ions such as zinc (Zn) and copper (Cu) are essential for dodder growth and metabolism, although an exceedingly high (over-homeostatic) supply of these micronutrients can result in growth inhibition and cellular toxicity. Even more so, non-essential metals such as cadmium (Cd), if transferred from the host to the parasite, need to be neutralized by timely detoxification mechanisms. In this work, we showed that *Cuscuta campestris* Yuncker establishes effective haustorial connections with leaf petioles and blades of *Daucus carota* L. (carrot), with the consequent transfer of Cd and essential metals (such as Zn and Cu) from the host vascular bundles to the parasite. Following up to this point, we detected the presence in the parasite of significant amounts of glutathione and phytochelatins, even in the absence of Cd exposure. This suggests that thiol peptides in dodder might be particularly important for Zn and Cu homeostasis as well as for Cd detoxification. Finally, we demonstrated that dodder is capable of synthesizing phytochelatins on its own, rather than massively importing them from the host, and also provided evidence for the existence of an endogenous, constitutively expressed, dodder's phytochelatin synthase.

1. Introduction

Cuscuta sp. (commonly named "dodder", belonging to the Convolvulaceae family) is a genus of obligate parasitic angiosperms capable of invading stems and leaves of a wide range of host plants (Dawson et al., 1994). Under favourable conditions, the rootless and leafless, yellowish-greenish thin dodder stem coils counterclockwise (sometimes clockwise) around the host and, at each point of contact, develops a haustorial structure within a few days (Vaughn, 2002, 2003). As soon as the haustorium has reached the host vascular bundles, it starts tapping water, photosynthates and mineral ions from the host (Malik and Singh, 1979; Jeschke et al., 1994, 1997; Birschwilks et al., 2006). Although it contains a few chloroplasts and small amounts of chlorophylls, and thus possesses an extremely low photosynthetic activity, dodder is believed to be completely dependent on its host for nutrient supply (Jeschke et al., 1994; van der Kooij et al., 2000; Birschwilks et al., 2006).

Dodder is considered a "super-sink" holoparasite that competes with developing fruits and other sink organs of the host by draining various kinds of assimilates and other products as well. It can virtually tap any kind of substance from the host's tissues, including viruses, phytoplasmas, secondary metabolites and xenobiotics (Wolswinkel, 1978; Malik and Singh, 1979; Dawson et al., 1994; Birschwilks et al., 2006). Thus, dodder should be endowed with prompt and sensitive regulatory mechanisms for handling homeostatic as well as excess levels of host-drained substances, in order to guarantee its own normal cellular metabolism.

Metals such as zinc (Zn) and copper (Cu) are essential for normal dodder growth and metabolism (Wallace et al., 1978; Malik and Singh, 1979; Boyd et al., 1999; Zhusupova, 2009), although an excessive, non-homeostatic supply of these micronutrients can result in growth inhibition and toxicity symptoms. Even more so, non-essential metals such as cadmium (Cd), if transferred from the host to dodder, are to be rendered less toxic by timely detoxification mechanisms. Thus, due to its holoparasitic nature and continuous sink activity, dodder is constantly confronted with a puzzle

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whereby, on one hand, it needs certain amounts of essential metal ions (such as Zn and Cu) for normal homeostasis; on the other, it has to protect itself from an excess of toxic metals such as Cd, which would otherwise cause irreversible damage and, eventually, death. This contrasting requirements could only be overcome by ensuring that excess/toxic metals are prevented from entering dodder cells and tissues, and/or by establishing a sensitive and efficient system of metal homeostasis and detoxification.

In this work we have taken into consideration the dodder species *Cuscuta campestris* Yuncker, parasitizing the host plant *Daucus carota* L. (carrot). The former was chosen because it represents a real threaten for many crops, and the most widespread dodder species worldwide (Dawson et al., 1994), included Italy (Benvenuti et al., 2005). However, in spite of its broad diffusion, several histo-anatomical and functional aspects of *C. campestris* parasitism have been overlooked until now, compared with other *Cuscuta* species, e.g., *C. reflexa*, *C. californica*, *C. europaea*, *C. nevadensis* and *C. odorata* (Wallace et al., 1978; Wolswinkel, 1978; Dawson et al., 1994; Boyd et al., 1999; Jeschke et al., 1994, 1997; Birschwilks et al., 2006). As to the carrot host, it was chosen because of its importance as a crop plant, its experimental tractability with regard to *in vitro* cultivation, and, not least, because of previous knowledge gained on thiol metabolism, especially regarding the response to Cd (Sanità di Toppi et al., 1998, 1999).

Thus, the aim of this work was, primarily, to demonstrate that *C. campestris* can indeed parasitize carrot plants by establishing effective haustorial connections, capable of transferring (or not) Cd and essential metal ions, such as Zn and Cu, from carrot vascular bundles to the parasite. Having proven the above, we subsequently raised the hypothesis that in "super-sink" plants such as *C. campestris*, the presence of glutathione (GSH) as well as of phytochelatins (PCs; Grill et al., 1985) might be particularly important for managing the homeostasis/detoxification of PC-inducing metals such as Cd, Zn and Cu; in fact, throughout its life-cycle, dodder is exposed to uninterrupted and concomitant fluxes of essential and non-essential metal ions coming from the host. Finally, we also wished to verify whether PCs could be synthesized by *C. campestris* on its own, rather than being massively tapped from the host.

2. Materials and methods

2.1. Plant material and growth conditions

D. carota L. (carrot) var. Berlicum seeds were sown in pots containing a mixture of peaty soil and inert sand, and grown in a greenhouse during the spring–summer period. The soil contained 1.06 and 0.07 $\mu g\,g^{-1}$ DW of bioavailable Zn and Cu, respectively (see Section 2.4). *C. campestris* Yuncker (dodder) seeds were scarified by immersion in concentrated sulphuric acid for 10 min, as in Benvenuti et al. (2005), rinsed three times with distilled water and placed in 9-cm Petri dishes with moistened filter paper, to permit seed imbibition. Seed germination was performed in a growth chamber at the constant temperature of 25 °C, in darkness for 5 d. 5-d-old dodder seedlings were placed in pots, close to 30-d-old carrot plants, and allowed to attach to the carrot leaf petioles or blades. At the same time, some carrot plants (placed in distinct pots) were kept non-parasitized by dodder.

2.2. Liquid culture and cadmium treatments

After 30 d of dodder parasitisation, the carrot plants ($n = 5$ per each replicate), abundantly rinsed with water, were placed in 250 mL flasks containing 100 mL of Gamborg's B5 medium each (Gamborg et al., 1968) (Sigma–Aldrich, Milan, Italy), pH 5.5. Gamborg's medium was chosen since, amongst the main standard nutrient solutions, it contains a fair SO_4^{2-} concentration (2.2 mM) and homeostatic levels of Zn and Cu (7.0 and 0.1 μM, respectively), and thus it is well-suited for experiments on metal effects in hydroponics. Flasks were put in a growth chamber at a constant temperature of 25 °C and a 16-h photoperiod. Half of the non-parasitized carrot plants and half of the parasitized ones were treated with 36 μM Cd for 2 and 4 d, whereas the others (non-parasitized and parasitized) were employed as untreated controls. At the same time, treatments with/without Cd were carried out in 250 mL flasks containing 100 mL of modified B5 medium (mB5), pH 5.5, identical to the above but lacking Zn and Cu.

At the end of the experiments, host and parasite plants were harvested, and carrot plants were dissected into roots, leaf petioles and leaf blades.

2.3. Histo-anatomy of dodder and carrot

Carrot leaf petioles and blades, tightly enwrapped by dodder stems, were cut into 1-cm segments and fixed in 70% (v/v) ethanol. The material was processed following dehydration method and embedded in Technovit 7100 resin (Heraeus Kulzer GmbH, Wehrheim, Germany). Serial transverse and longitudinal sections 8-μm-thick were made using a Leitz 1512 rotation microtome (Haxagon Metrology GmbH, Wetzlar, Germany), stained with Toluidine Blu O and mounted in Eukitt (Bio-Optica, Milan, Italy). The sections were photographed by a Leica DM4000 B microscope equipped with a Leica DC100 digital camera (Leica Microsystems, Wetzlar, Germany).

2.4. Heavy metal determination

The Cd, Cu and Zn concentrations in samples of carrot and dodder (and in the peaty soil, as far as the bioavailable metal fraction is concerned) were measured by inductively coupled plasma-atomic emission spectrophotometry (ICP-AES).

Prior to ICP-AES analysis, plant samples were oven-dried overnight at 80 °C and ground to a powder. One hundred mg of each sample was added to 2.5 mL of 65% (v/v) aqueous HNO_3 and 0.5 mL of 30% (v/v) H_2O_2, and then digested in a Milestone MLS-1200 MEGA microwave system (FKV, Sorisole, Italy), equipped with temperature control and high pressure vessels. Before the ICP-AES analysis, digested samples were diluted with 10% (v/v) HNO_3 to a final volume of 10 mL.

An ULTIMA 2 instrument (Jobin Yvon, Longjumeau, France), equipped with a Meinhard nebulizer and a cyclonic spray chamber, was used for the ICP-AES analysis. The wavelengths used, set by analyzing a 100 mg L^{-1} standard solution of metals, were respectively 228.802 nm for Cd, 224.700 nm for Cu, 213.856 nm for Zn.

2.5. Thiol-peptide separation, detection and quantification

Samples of carrot and dodder (400 mg FW for each), exposed to 36 μM Cd for 2 and 4 d, were homogenised in a mortar in ice-cold 5% (w/v) 5-sulfosalicylic acid (SSA), containing 6.3 mM diethylenetriaminepentaacetic acid (DTPA), according to de Knecht et al. (1994). After centrifugation at 10,000 × *g* for 10 min at 4 °C, the supernatant fraction of extracts was filtered through Minisart RC4 0.45 μm filters (Sartorius, Goettingen, Germany) and immediately assayed by HPLC (Model 200, PerkinElmer, Wellesley, US-MA), following again de Knecht et al. (1994). Thiol-containing peptides (GSH and PCs) were separated by a Purosphere reverse-phase C_{18} column (Merck, Darmstadt, Germany), by injecting 200 μL of extract. The separation was obtained by using a 0–26% CH_3CN gradient, with a flow rate set at 0.7 mL min^{-1}. The elution solutions contained 0.05% trifluoroacetic acid (TFA). Thiol-containing peptides were determined using post-column derivatization with 300 μM Ellman's reagent [5,5′-dithio(2-nitrobenzoic acid)], detected at 412 nm and quanti-