	Experiment title: **Sub-cellular localization of Cd in plant tissues**	**Experiment number**: EC-719
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Report:

Cd is extremely toxic even at low concentrations, and it is easily taken up by plants and transferred to the food chains thus presenting risk for animal and human health [1]. The use of plants that are able to take up extreme metal concentration (so called hyperaccumulators) had appeared as promising way for metal removal from soil; however, more knowledge on plant metal accumulation and detoxification mechanisms is needed before they can be successfully introduced into phytoextraction applications [2, 3]. The differences in metal compartmentalisation on tissue, cellular and sub-cellular levels are one of the basic tolerance mechanisms in these plants, so localization studies are of fundamental importance to clarify and understand them. In this study we used μ-XRF imaging and μ-XANES to obtain direct information on localization and chemical state of Cd in tissues of Cd hyperaccumulator *Thlaspi praecox* grown in nutrient solution supplied with 100 μM Cd as $CdSO_4$ or $CdCl_2$. In preliminary study we have found out that plants supplied with $CdSO_4$ accumulated significantly higher Cd concentrations in their leaves then plant supplied with $CdCl_2$. The observation that chemical form of Cd added to the nutrient solution influences the level of Cd accumulation is novel, so studies of localization and chemical state of Cd in leaves and roots were aimed to resolve the mechanisms of accumulation and tolerance of Cd at tissue, cellular and sub-cellular level with the possible involvement of sulphur metabolism.

Within the obtained beam-time we have managed to map Cd and simultaneously S, Cl, Mg and Zn using zone plate micro-focusing (excitation energy of 3.55 keV) in different leaf regions (epidermis, mesophyll and veins) of *T. praecox* plants treated with 100 μM Cd as $CdSO_4$ or $CdCl_2$ added to the nutrient solution. In the regions with high Cd concentrations μ-XANES spectra were recorded using zoneplate micro-focusing, while in tissues with lower Cd concentrations (e.g. veins) XANES spectra were recorded using 60 μm pinhole. Pure thin-foil standard reference materials were measured in order to calibrate XRF system and quantify the results obtained by μ-XRF mapping using the software, which is under development in our research group. In addition XANES spectra of reference Cd compounds ($CdSO_4$, CdS, $CdCl_2$, Cd-pectinate, Cd-celulose, Cd-glutathione) in form of solid pellets were measured using 60 μm pinhole.

Plant samples were prepared in our laboratory from plants grown in nutrient solution with added 100 µM Cd as $CdSO_4$ or $CdCl_2$ using cryo-fixation, cryo-sectioning at -30 °C and freeze-drying at -30 °C and 0.340 mbar. The sections were mounted in a sandwich between two Al holders with 12 2 mm holes and 2 layers of 4 µm ultralene and attached to the target holder of the SXM. In order to perform the comparison of Cd localization in leaves on (sub)cellular level experimental methods with increased sensitivity are required since the absolute concentrations of Cd in different dry plant tissue samples grown in controlled environment vary around 500 ppm. In addition, superior lateral resolution is needed in order to enable localization at the sub-cellular level. Such lateral resolution is easily achieved by the SEM-EDS technique but in this case we are limited by the detection limits, typically in the order of 0.1%, which is not enough to perform localization of metals in trace amounts (few 10 to few 100 ppm) which is required in our study. The Scanning X-Ray Microscope (SXM) at the ID21 beamline is perfectly suitable to perform the proposed study providing the sensitivity down to ppm level combined with excellent lateral resolution and has been already used successfully for metal localization in plant tissues [4]. In addition, it enables also the investigation of chemical form(s) of Cd employing the µ-XANES spectroscopy. Compared to µPIXE or SEM-EDS where Cd signal (L3 line at 3.134 keV) partially overlaps with the strong neighbouring K signal (Kα line at 3.314 keV), which makes Cd mapping almost impossible, this can be avoided in the synchrotron experiment by tuning the beam between the Cd L3 edge (3.538 keV) and potassium K edge enhancing significantly the sensitivity for the Cd detection.

The X-ray beam delivered by the undulator was monochromatized by Si(111) double crystal monochromator and focused to a submicron probe (0.3 x 0.7 µm^2) using zoneplate micro-focusing. The fluorescence emission of the sample was collected by a SDD detector. The excitation energy for the scan was set to 3.55 keV (i.e above the Cd- L3 edge and below the potassium K edge in order to get rid of the strong K signal) to record maps of Cd, Cl and S simultaneously. Finally the L3 – edge XANES spectra of Cd were recorded in the region from 3.50 to 3.66 keV in selected positions of apoplast and symplast of epidermal and mesophyll cells. Obtained XRF spectra were processed using PyMCA software.

The preliminary results showed that in leaves the pattern of Cd accumulation was similar in both treatments (maps of $CdCl_2$ treatment not shown). Cd accumulated mainly in vacuoles of epidermal cells. In epidermal cells a strong colocalization with Cl was observed (Figs. 1a and b), while no correlation with S localization was found (Fig. 1c). In mesophyll cells, however, Cd colocalization with Cl was observed especially in the cell walls (Figs. 2a and 2b) and colocalization with sulphur was also observed in symplast (vacuoles) (Fig. 2c). One has to be aware, however that colocalization of two elements does not necessary mean that the two elements are actually coordinated to each other, therefore µXANES spectra were recorded in marked (X) positions (Figs. 1A and 2A).
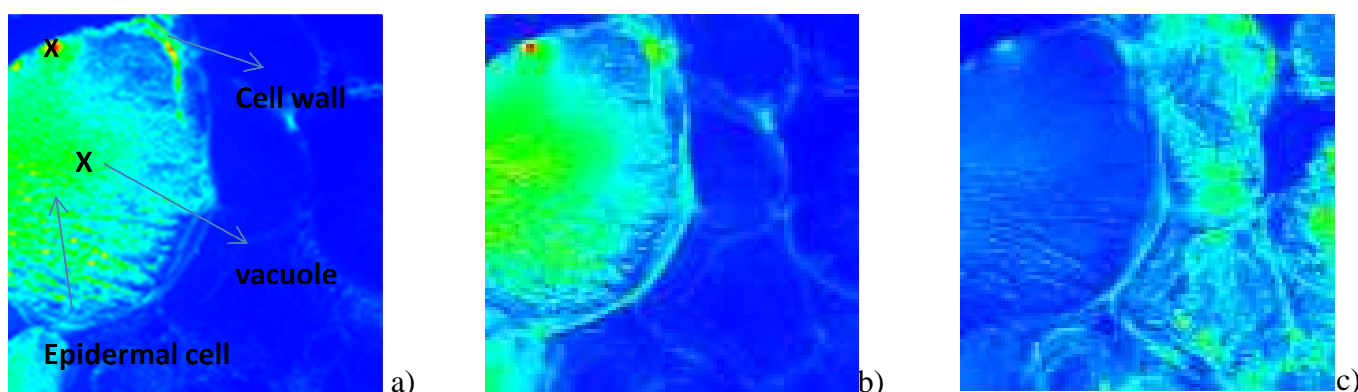

Figure 1: Epidermal-mesophyll region of plants treated with 100 µM $CdSO_4$. Elemental maps 100x100 µm^2, with 1 µm^2 resolution. a) cadmium, b) chlorine, c) sulphur.

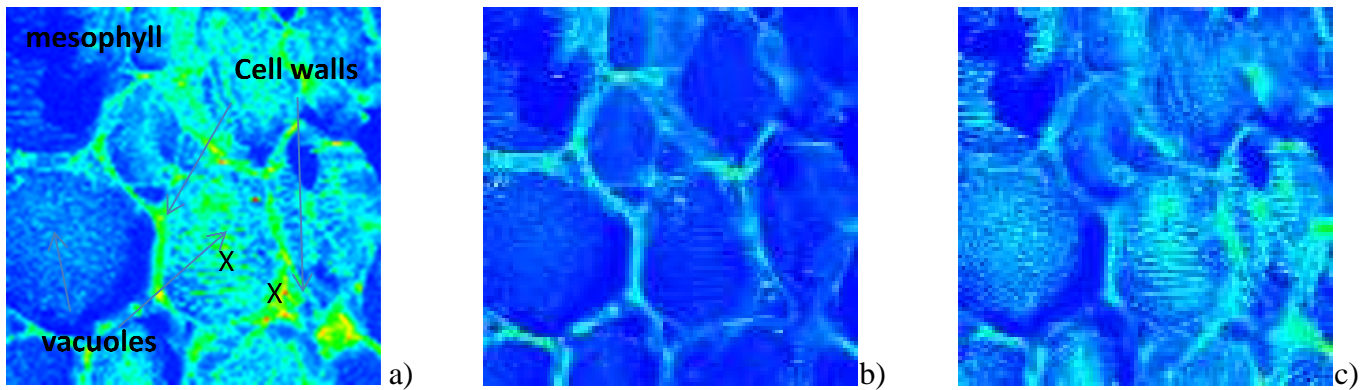**Figure 2**: Mesophyll region of plants treated with 100 µM $CdSO_4$. Elemental maps 100x100 μm^2, with 1 μm^2 resolution. a) cadmium, b) chlorine, c) sulphur.

Fig. 3 shows Cd L3-edge XANES spectra of reference compounds: Cd-glutathion, Cd-pectinate and $CdCl_2$ complexes. In the first complex Cd is bound to S (Cd-S-C coordination) and in second Cd is bound to oxygen (Cd-O-C coordination). In $CdCl_2$, Cd is bound directly to chlorine.
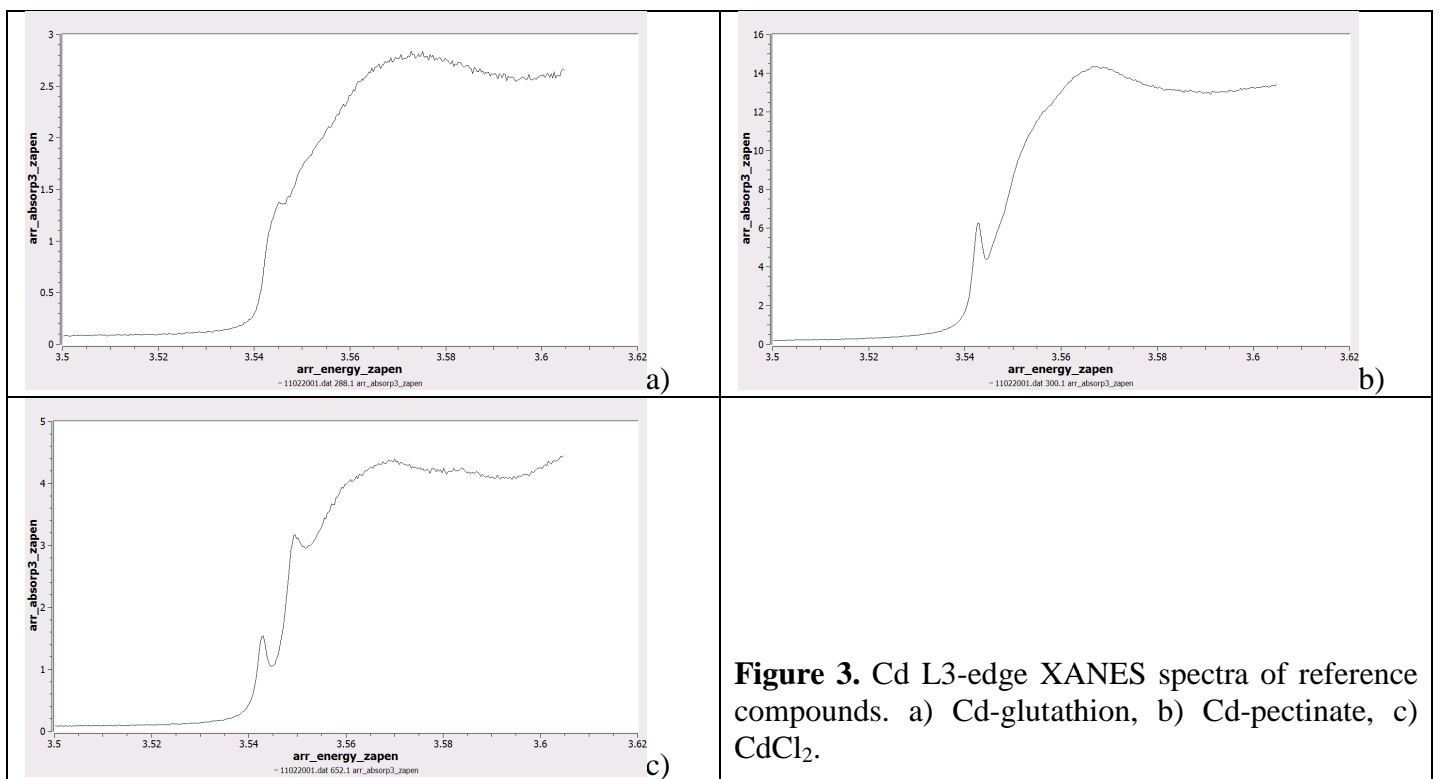

Figure 3. Cd L3-edge XANES spectra of reference compounds. a) Cd-glutathion, b) Cd-pectinate, c) $CdCl_2$.

When comparing XANES spectra of the reference compounds (Fig. 3) to those measured in plant tissues (Fig.4), XANES spectrum recorded in epidermal cell walls and in symplast (Fig. 4a) is similar to that of Cd-pectinate, indicating that Cd is bound mainly to oxygen ligands (Fig. 4b), however a coordination to Cl cannot be ruled out without further quantitative analysis, especially because of high colocalization of Cd and Cl found in epidermal tissues. In mesophyll, XANES spectra indicated that Cd is coordinated mainly to sulphur ligands (Figs. 4c and 4d). XANES spectra measured in leaf tissues and cell parts of the plants treated with $CdCl_2$ exhibit the same characteristics and are therefore not shown. In veins no distinct differences in Cd ligands were observed between the two treatments, indicating that mechanisms at the root level probably govern the level of Cd accumulation in shoots. However a detailed further analysis using linear combination fit of XANES spectra will give us more precise information about chemical state of Cd in particular tissues and cell parts.
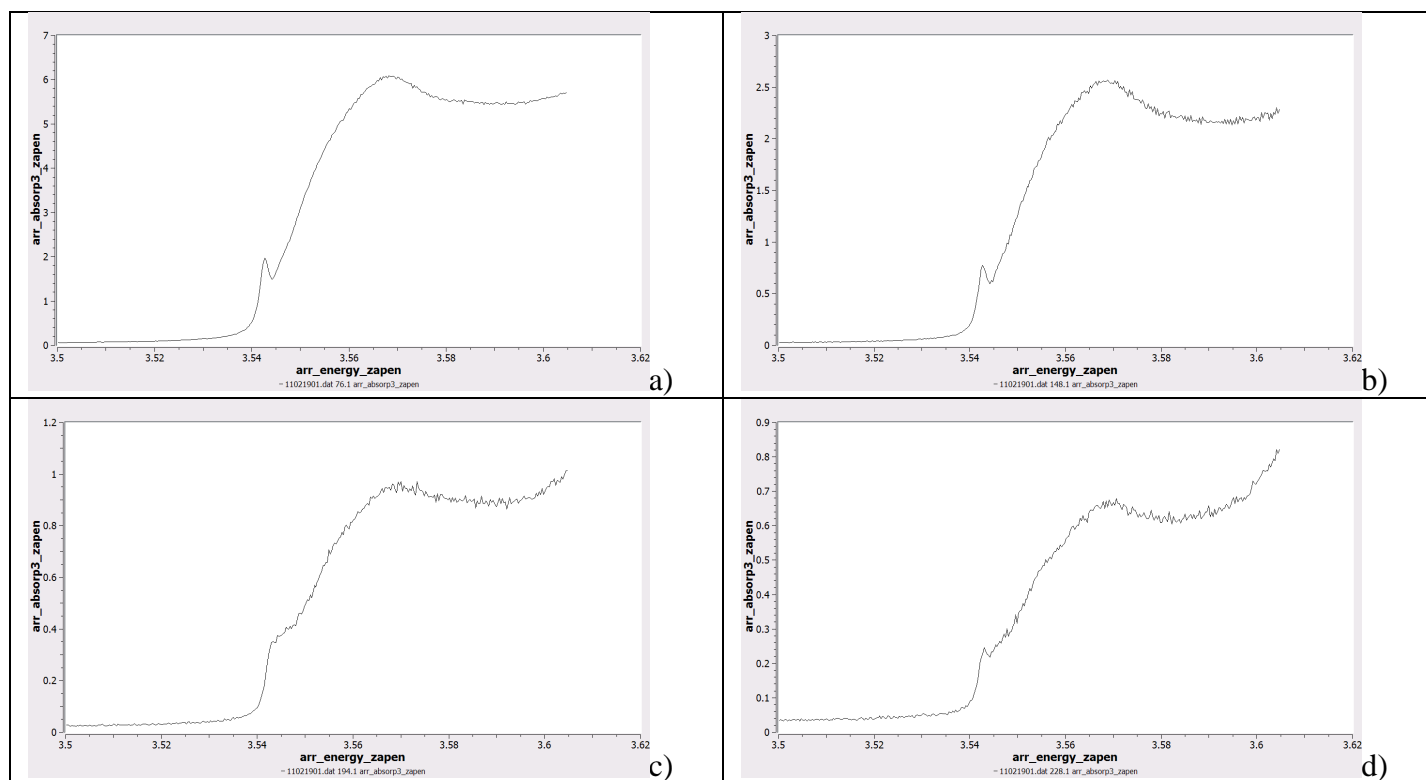
Figure 4. Cd L3-edge XANES spectra measured in epidermal tissues a) cell wall, b) symplast; and mesophyll tissues c) cell wall, d) symplast.

In conclusion, the preliminary results show, that in epidermal cells Cd distribution intensively correlates with that of Cl, and that Cl cannot be ruled out as potential ligand for Cd, which represents a novelty. In similar previous studies Cd was found to be linked to sulphur and oxygen ligands [4, 5]. We have also shown that different Cd ligands may exist in epidermal and mesophyll cells, which was also not known before [4, 5]. In veins of plants treated with $CdSO_4$ and $CdCl_2$ there are no distinct differences in chemical state of Cd, so we conclude that the mechanisms of uptake, compartmentalization, complexation and transport at the root level probably govern the level of Cd accumulation in shoots. Therefore, further studies on localization and chemical forms of Cd as well as involvement of sulphur compounds in different root parts of *T. preacox* are needed to understand the differences in Cd uptake between $CdSO_4$ and $CdCl_2$ treated plants.

References

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