Development of a rapid biolistic assay to determine changes in relative levels of intracellular calcium in leaves following tetracycline uptake by pinto bean plants†

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Tetracycline antibiotics, such as chlortetracycline (CTC) and tetracycline (TC), are introduced into agricultural lands through the application of manure as fertilizer. These compounds are phytotoxic to certain crop plants, including pinto beans (*Phaseolus vulgaris*), the species used for this investigation. While the mechanism of this toxicity is not yet understood, CTC is known to be a calcium chelator. We describe here a novel method to show that CTC is taken up by pinto bean plants and chelates calcium in leaves. Cameleon fusion proteins can provide qualitative and quantitative imaging of intracellular calcium levels, but current methodology requires stable transformation. Many plant species, including pinto beans, are not yet transformable using standard *Agrobacterium*-based protocols. To determine the role of calcium chelation in this plant, a rapid, biolistic method was developed to transiently express the cameleon protein. This method can easily be adapted to other plant systems. Our findings provide evidence that chelation of intracellular calcium by CTC is related to phytotoxic effects caused by this antibiotic in pinto beans. Root uptake of CTC and TC by pinto beans and their translocation to leaves were further verified by fluorescence spectroscopy and liquid chromatography/mass spectrometry, confirming results of the biolistic method that showed calcium chelation by tetracyclines in leaves.

Introduction

Intracellular calcium is undeniably an important component of a eukaryotic cell's ability to physiologically respond to stimuli, both internal and environmental.[1,2] To understand the role of calcium signaling in response to stimuli, it is very important to evaluate calcium fluxes within cells. Many methods exist for measuring intracellular calcium, including calcium-sensitive dyes and calcium-binding fluorescent fusion proteins. Cameleon fusion proteins are commonly used for calcium detection; these consist of two modified green fluorescent proteins (cyan fluorescent protein, CFP, and yellow fluorescent protein, YFP) linked *via* a calmodulin calcium-binding site and an M13 peptide linker sequence.[3] This design allows for comparative measurement of calcium *via* fluorescence resonance energy transfer (FRET). FRET occurs when calcium binding causes condensation of the calmodulin domain around the M13 peptide sequence, enabling direct interaction and energy transfer between the CFP and YFP components.

The advantage to using the cameleon protein for calcium quantitation is that it can be stably and constitutively expressed in nearly all cell types, and can be targeted to the cytoplasm or to specific organelles.[3–5] Expression in plants typically involves using *Agrobacterium tumefaciens* to transform plant cultures.[5] This process requires aseptic regeneration and propagation from transformed plant callus on culture media, to ultimately produce viable intact plants that express the cameleon protein in all of their tissues. While this technique is widely used and very effective, it can also be problematic, in that not all plant species are transformable. In addition, transformation efficiency, gene silencing, and variable expression throughout development can lead to difficulties in the reliability/reproducibility of data retrieval.[5,6] Transformation protocols must be established for each plant species and, often, each plant cultivar.[7,8] Finally, while calcium-sensitive dyes are often used to determine relative calcium levels, microinjection protocols used to introduce dyes into the plant tissues can be accompanied by leakage from the plant cells, and lead to cell death up to as high as 75%.[5]

To overcome these difficulties, we utilized a novel biolistic approach for introducing the cameleon plasmid YC3.6 into pinto bean leaves (Fig. 1).[9] The versatility of biolistic transient expression allows this protocol to be adaptable to all plants, as well as other organisms, and virtually any tissue or cell type, for rapid comparative analysis of calcium levels. To demonstrate the reliability of this methodology, pinto bean plants were subjected to treatments leading to both increases and decreases in intracellular calcium levels. Increases were induced with calcium chloride ($CaCl_2$); reductions were induced using the calcium-chelating ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetra-acetic acid (EGTA).

This biolistic method was developed to define mechanisms of chlortetracycline (CTC) toxicity in a non-transformable plant

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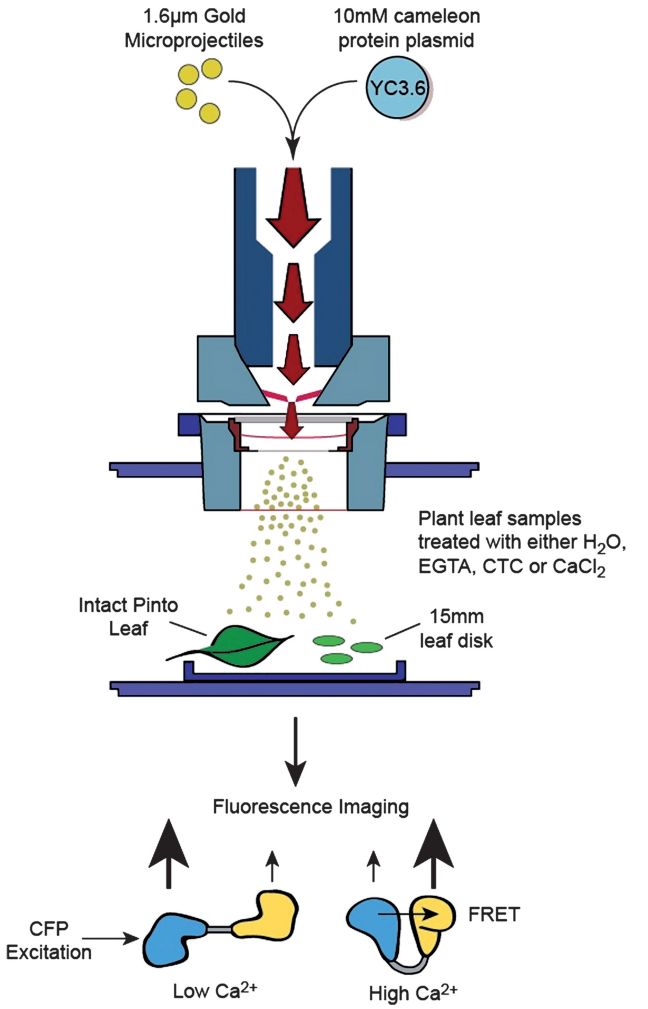

Fig. 1 Experimental design. YC3.6 plasmid DNA coated onto microprojectiles was biolistically transformed into leaf tissues. After allowing for recovery and expression, relative calcium levels were determined by FRET analysis of transformation foci. With low calcium, CFP and YFP components of YC3.6 do not interact, giving low YFP/CFP ratios under CFP excitation. At high calcium the components interact, causing high YFP/CFP ratios. Since comparative determinations of calcium levels are based on YFP/CFP emission ratios from abundant, constitutively produced YC3.6, differences in size, number of transformed cells, or overall expression levels between foci did not significantly affect variation in data collected.

species. The CTC antibiotic is a known calcium-antagonist[10] with unknown effects on intact tissues of pinto beans. CTC is an environmental pollutant primarily from agricultural sources, and phytotoxic to pinto beans, resulting in deficient growth and nutrient uptake. It was originally hypothesized that CTC was toxic to the root-associated rhizobia and not to the plant itself,[11] although we have observed toxicity with non-inoculated pinto beans grown in sterilized soil.[12] We have also noted that unlike some plants, pinto beans are unable to detoxify this antibiotic. Calcium is known to be a primary regulator that affects many aspects of plant development, and deficiencies in calcium concentrations can lead to inhibition of growth.[13] The experiments presented here test the hypothesis that CTC is readily taken up by pinto bean plants and chelates intracellular calcium leading, at least in part, to the phytotoxic effects observed with this plant.

Experimental

Plant treatment for biolistic transformation

Pinto beans (*Phaseolus vulgaris*) were germinated in artificial soil (prepared in-house). To develop and optimize our experimental conditions, two variations of treatment were performed prior to biolistic transformation. To ensure rapid uptake, the first experiment utilized 15 mm YC3.6-expressing leaf disks, excised from bombarded leaves (see below) and placed for 1 h on 200 μL of the following solutions: 5 mM chlortetracycline (CTC), 5 mM ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 10 mM calcium chloride ($CaCl_2$), and distilled H_2O. The second experiment treated whole plants for 24 h in the same spiked water solutions prior to bombardment, except that 0.250 mM CTC and 0.5 mM EGTA were used because of toxicity to the intact plants.

Biolistic transformation

Bombardment entailed detaching leaves (6–8 cm in length) from 10-day-old pinto bean plants and placing them onto a Petri dish lined with H_2O-moistened filter paper (Whatman 3MM). Two leaves were used per dish. For particle bombardment, gold particles (1.6 μm in size) were washed, prepared, and coated with YC3.6 cameleon plasmid DNA according to manufacturer's protocols (Biolistic PDS-1000/He Particle Delivery System, Bio-Rad). Leaves were bombarded once at 900 psi using a PDS-1000/He Particle Delivery System (Bio-Rad), according to methods described previously.[14] Each bombardment was performed in triplicate using 10 μL gold/plasmid DNA suspension, using macrocarriers and stopping plates (Bio-Rad) placed ~12 cm from the leaves. After bombardment, the leaves were incubated in the dishes with illumination at 25 °C for 18 h and analyzed by microscopy.

Fluorescent microscopy

Fifteen millimeter leaf disks were excised from YC3.6-expressing portions of intact leaves and placed in a depressed microscope slide. Two hundred microliters of distilled H_2O was placed on top of the leaf disk followed by a cover slip, which was sealed with a paraffin-based wax to prevent dehydration of the leaf disk during microscopy. Fluorescence resonance energy transfer (FRET) analysis of transformation foci observed on the leaf surface was performed using a high resolution Zeiss Axioplan 2 fluorescent microscope (Zeiss, Hamburg, Germany) equipped with a FRET filter system for detecting both CFP and YFP emission (480 nm and 535 nm, respectively) from 435 nm excitation. Digital images captured at 40× magnification were analyzed using ImageJ software.[15] Each treatment used in this study included FRET analysis of captured images for at least 50 CFP/YFP-expressing transformation foci from three separate leaf disks.

^{3}H-Tetracycline plant uptake

In a separate experiment to determine antibiotic uptake by pinto beans, plants were treated with radiolabeled ^{3}H-labeled TC. Plants grown after 10 days post-germination were removed from their containers, and washed thoroughly with distilled H_2O to remove soils from the roots. Four pinto bean plants were placed in 300 mL distilled H_2O spiked with 2 μCi of ^{3}H-labeled TC (American Radiolabeled Chemicals, Inc., St. Louis, MO). After 24 h of treatment, 130 mg of leaf tissue were removed from the uppermost leaves and homogenized using a glass dounce homogenizer in 1 mL distilled H_2O. The homogenized leaf mixture was decanted into 9 mL of liquid scintillant (National Diagnostics, Atlanta, GA) and radioactivity was counted using a Tricarb 1600TR liquid scintillation counter (PerkinElmer, Waltham, MA).

Plant treatment

Post-germinated (12- to 14-day old) pinto beans were used to determine chlortetracycline (CTC) and tetracycline (TC) uptake from soil. The plants were divided into three set-ups: (a) plants treated with 0.25 mM CTC solution (CTC-treated), (b) plants treated with 0.25 mM TC (TC-treated), and (c) untreated plants that received only distilled water (control). The plants were exposed to the antibiotics for 24 h; then, leaves from the treated and untreated plants were harvested and extracted.

Approximately 1.0 g of leaf material from each plant was macerated and ground with 10 mL (2 : 1 : 1, v : v : v) methanol–McIlvaine buffer–0.020 M EDTA in a Kontes Duall tissue grinder. The extracts were clarified by centrifugation in a Mini Spin Eppendorf centrifuge at 10 000 rpm for 4 minutes and filtered through a 0.45 μm nitrocellulose membrane. An aliquot of the plant extract (500 μL) was adjusted to 3 mL in carbonate buffer (pH 10, 100 mM) and stored for 20 h at 23 °C. The resulting solution was then analyzed using a spectrofluorimetric method (see below). In addition, 250 μL of the extract was adjusted to 1 mL with deionized distilled water and analyzed by liquid chromatography/mass spectrometry (LC/MS) (see below). Emission spectra and mass spectra of CTC and TC standards were also collected for comparison of results.

Fluorescence measurements and LC/MS analysis

Fluorescence emission spectra of CTC and TC in plant extracts and standards were recorded using an SLM model 8100 spectrofluorimeter equipped with a 450 W Xe arc lamp source. The excitation and emission spectral band-pass were set at 4 nm for all experiments. The excitation wavelength used was 355 nm and the emission from 370 to 600 nm was recorded. Three readings were obtained for each of the samples and standards. All measurements were corrected by subtracting the emission spectra of blank samples.

The plant extracts from the three treatments were also analyzed by LC/MS using an LCQ Advantage™ ion trap mass spectrometer connected to a Surveyor LC system (Thermo Finnigan, San Jose, CA, USA), operated using electrospray ionization in positive mode. A reversed-phase Thermo Hypersil-Keystone (Bellefonte, PA, USA) BetaBasic C18-column (100 × 2.1 mm internal diameter with 3 μm particle size) was used for separation. The capillary temperature was 200 °C, capillary voltage was 10 V, and the spray voltage was 4.5 kV for all applications. Nitrogen was used as sheath gas at a flow rate of 20 μL min^{-1}, and helium gas was used to induce dissociation of selected ions using 48% normalized collision energy. A gradient mobile phase starting at 5% acetonitrile and 95% water (with 0.3% formic acid) (held for 3 min) was used; the mobile phase changed using a linear gradient to a final composition of 95% acetonitrile and 5% water (with 0.3% formic acid) within 10 min. This composition was maintained for 1 min, then the mobile phase was returned to the initial condition. The flow rate was 200 μL min^{-1}, the column temperature was 30 °C, and the full loop injection volume was 20 μL.

Results and discussion

Our earlier studies showed that TC-associated phytotoxic effects occur in rhizobium-free pinto bean plants that have been exposed to CTC.[12] Thus, we hypothesized that TC-related antibiotics are taken up through the roots into the plant, directly leading to phytotoxicity. In fact, our initial studies using ^{3}H-labeled tetracycline (TC) demonstrated direct evidence of antibiotic uptake by the pinto bean plants. This experiment was conducted by germinating pinto beans in sterile artificial soil that was not inoculated with a rhizobium culture. At 10 days post-germination, these rhizobium-free seedlings were treated with ^{3}H-labeled TC. Radioactively labeled CTC is not commercially available; however, for the purpose of uptake TC is a suitable alternative due to its structural and chemical similarities. We found after 24 h of treatment that approximately 29% of the radioactive TC was found in the leaves of the pinto beans (Table 1). These data are significant because they provide evidence that a tetracycline antibiotic can be used for uptake studies, and demonstrated efficient uptake/transport of TC from roots to the leaves. Based on these initial findings, we hypothesized that CTC, as used in our previous studies,[12] is taken up and incorporated into the plant cells beyond the level of the roots and the rhizosphere.

The biolistic transformation protocol used for this study is illustrated in Fig. 1. To determine if CTC could chelate calcium within leaf cells *in vivo* after being taken up by the pinto bean plants, two approaches were used for biolistic transformation of the cameleon protein. In each case, intact leaves were used for bombardment to ensure that the greatest number of particles would hit the leaf surface, and maintained leaf tissue viability prior to treatment and analysis. To determine the sensitivity of the assay, initial concentrations of reagents used to increase or decrease calcium were relatively high, to aid in the rapid uptake

Table 1 Percentage of ^{3}H-labeled tetracycline uptake in leaf tissue of pinto beans

Plant #	Leaf fresh weight (g)	% Uptake/g tissue
1	1.85	27.69
2	1.70	26.92
3	1.95	29.23
4	2.10	32.31
	Average ± SD	**29.04 ± 2.38**

into the leaf disks. FRET imaging and qualitative analysis of these transformation foci indicated that this assay is sensitive and reliable enough to detect differences in calcium levels in pinto bean leaf disks exposed to the different treatments. Fig. 2A and 2B show representative visual images of foci from leaf disks treated with distilled H_2O (controls). Notably, this image depicts many foci observed within an area of approximately 20 μm^2, with very low autofluorescence background. Fig. 2C and 2D show foci from leaf disks treated with CTC. Again, numerous foci were observed, indicating efficient transformation and expression under various treatments. Bombardment can lead to irreparable cell damage, but an advantage of this method with respect to biolistic loading of calcium-sensitive dyes is that cell viability can be observed *via* cameleon-expressing cells. Among its other pitfalls, cell viability cannot be determined with calcium-sensitive dyes.[5]

Under some circumstances, CTC can be used as a fluorescent Ca^{2+} indicator. With an excitation wavelength of 390 nm and emission wavelength of 530 nm,[16,17] it must be considered that this antibiotic itself could potentially have an effect on fluorescence measurements. However, while the emission spectra might be within the range of CFP (535 nm), the excitation wavelength was much lower than that used for this assay (435 nm). Oliver *et al.*[18] also noted that at room temperature the fluorescence of CTC is reduced by 50% of its maximum. Our fluorescence measurements detected no observable difference between the background fluorescence of plants treated with CTC, relative to the control plants (as measured from non-transformed regions outside of the YFP/CFP-expressing foci). Thus our data indicate that fluorescence measured from the cameleon-expressing foci is significantly higher than the negligible (*i.e.* undetectable under these conditions) background resulting from CTC uptake in treated leaves. Thus CTC fluorescence was not a factor in the comparative FRET determinations used for this study.

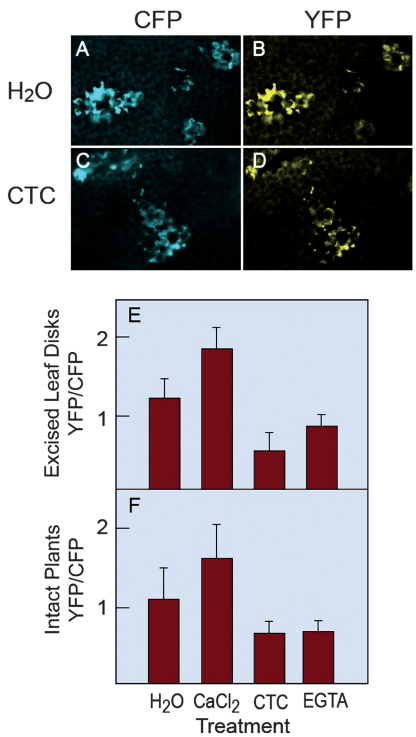

Fig. 2 Imaging and FRET analysis of biolistic transformation foci expressing cameleon protein in pinto bean leaves. **A** and **B** show CFP and YFP emission, respectively, both from the wavelength for CFP excitation, for representative foci on an H_2O-treated leaf. **C** and **D** show the same emissions from foci on a CTC-treated leaf. Images were captured using a 40× objective. **E** and **F** show YFP/CFP ratios for excised leaf disks and intact plants, respectively, exposed to the various treatments indicated.

The YFP/CFP (535 nm/480 nm) ratio is the unit of measure for determining relative calcium levels. A YFP/CFP ratio above 1.0 would suggest that a majority of cameleon protein molecules have bound intracellular calcium, resulting in FRET. This is observed for the $CaCl_2$-treated leaf disks (Fig. 2E). Control plants treated with H_2O would be expected to have an overall YFP/CFP ratio of approximately 1.0, as observed in our experiment. This average is expected due to varying levels of calcium occurring throughout the different leaf cell types.[19] Low calcium levels would result in YFP/CFP ratios less than 1.0, as is observed in the EGTA- and CTC-treatments. Relative to the H_2O control, the YFP/CFP ratios for $CaCl_2$, EGTA, and CTC treatments were significantly different ($p < 0.05$), demonstrating that this biolistic-based method can clearly differentiate the effects of various treatments on calcium levels in pinto bean leaves. Based on these FRET measurements, we can conclude that reductions in levels of intracellular calcium do in fact occur following CTC treatment of pinto bean leaf disks.

A second approach was used to demonstrate that this method can be used for detecting calcium level changes when intact plants are exposed to various treatments, prior to leaf detachment. These experiments were again based on the hypothesis that CTC uptake leads to calcium chelation *in vivo*, providing one causative mechanism for phytotoxicity. Ten-day-old pinto bean plants were transplanted into separate containers of distilled H_2O, 10 mM $CaCl_2$, 0.5 mM EGTA, and 0.25 mM CTC, and treated for 24 h to allow optimal uptake by roots into the plants. Lower EGTA and CTC concentrations were used, since the higher concentration used for leaf disks caused lethality in intact plants within hours. Immediately following treatment, leaves were detached, biolistically transformed, and analyzed for FRET

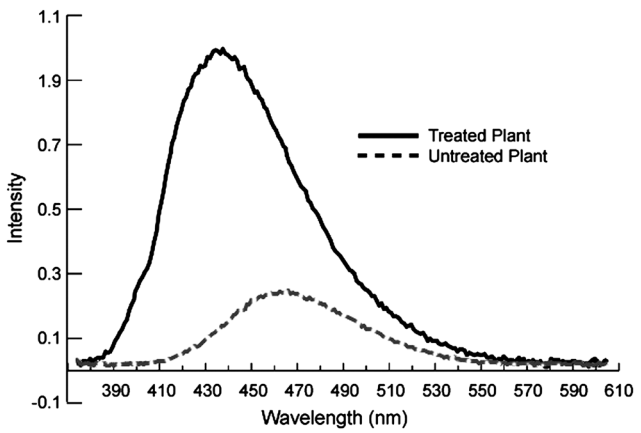

Fig. 3 Emission spectra of extracts from CTC-treated and untreated plants.

after an 18-h incubation, using the optimized conditions determined for the leaf disks. Fig. 2F shows the YFP/CFP ratios obtained for control and treated intact plants. These data correlate closely with those obtained from the treated leaf disks, with YFP/CFP ratios significantly greater than 1 for $CaCl_2$-treated plants, and less than 1 for EGTA- and CTC-treated plants. In fact, standard deviations were reduced with foci from the intact plants, suggesting that this is a viable and useful method for determining the effects of environmental contaminants on calcium flux in soil-grown plants. This method can be applied to a variety of abiotic treatments, using greenhouse- as well as field-grown plants.

Taken together, the previous experiments strongly suggest that CTC uptake, and calcium chelation within leaf cells of the pinto

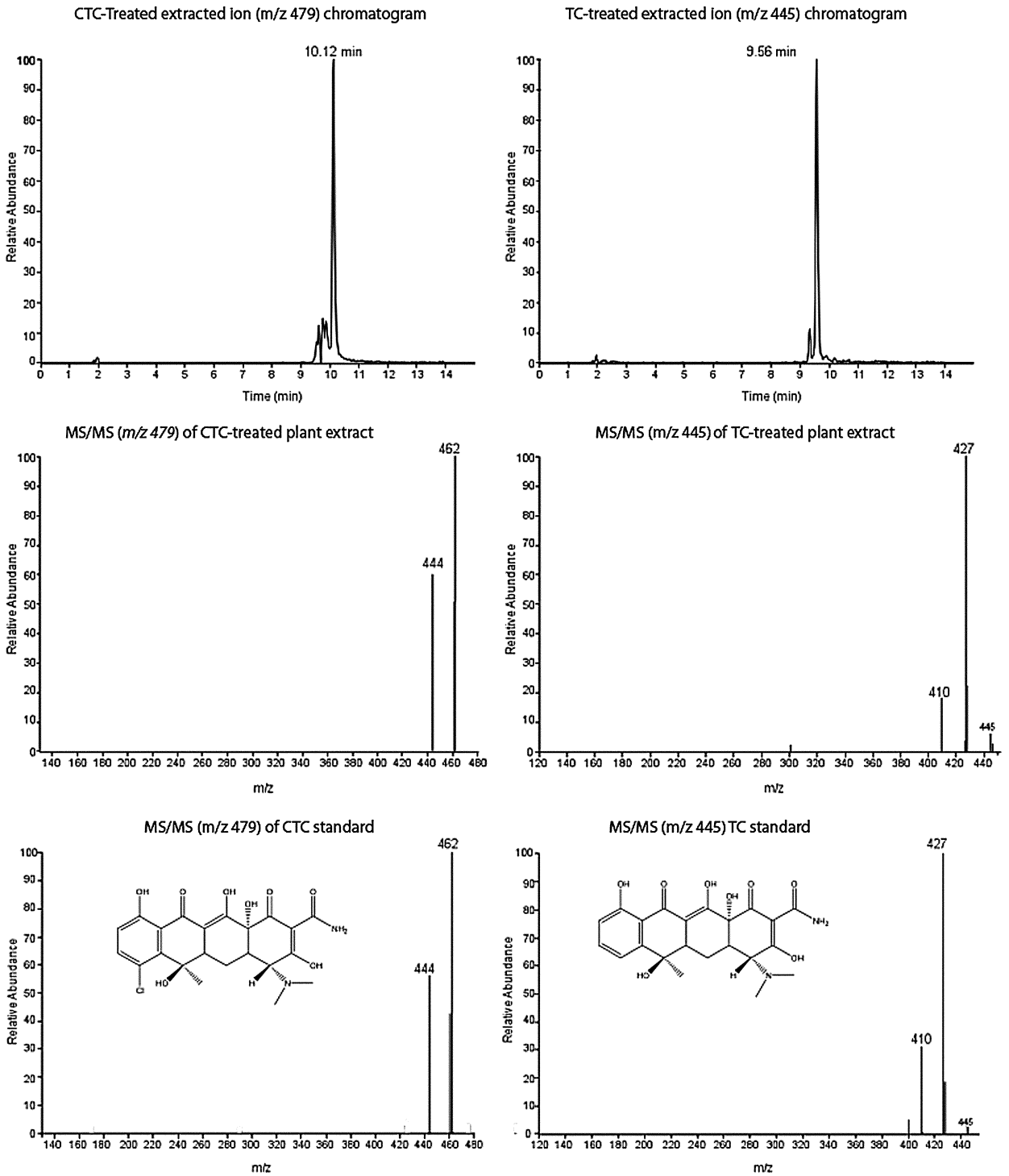

Fig. 4 Extracted ion chromatogram ($m/z = 479$ for CTC and $m/z = 445$ for TC) of CTC-treated (top left) and TC-treated (top right) plant samples. The MS/MS spectra of the treated plant samples and the standards are shown in the middle and bottom panels (CTC in the left and TC in the right).

bean plants, may be associated with observed phytotoxic effects. To provide further support for this hypothesis, it was important to confirm that CTC itself, like TC, could be efficiently taken up by these plants. Toward this end, two types of analytical experiments were conducted to directly confirm uptake of these antibiotics by the plants.

The first approach is based on spectrofluorimetric measurements of isochlortetracycline (iCTC), the preferred isomeric form of CTC in basic pH. While the fluorescence of CTC is enhanced by the addition of calcium ions,[16] and fluorescence measurement of the CTC-Ca^{2+} complex has been the basis of several studies,[20,21] higher fluorescence intensity can be obtained by converting CTC to iCTC.[22] Hence, the plant extracts and CTC-Ca^{2+} standard were added to a carbonate buffer solution (pH 10, 100 mM). It is known that iCTC is ineffective in chelating calcium ions[23] and by adding EDTA in the extracting solution, CTC is released from the CTC-Ca^{2+} complex because Ca^{2+} preferentially binds to EDTA. At this pH, the unbound CTC is readily converted to iCTC.

Results from the emission scans of the treated and untreated plant extracts are shown in Fig. 3. A maximum peak around 435 nm was observed in the CTC-treated plants. This is the signature emission region of iCTC,[16] providing direct evidence that the CTC was taken up by the pinto beans. The lower intensity and broad peak observed in the 410–540 nm region in the untreated plant extract is due to fluorescence from the sample matrix, which caused a slight red shift on the iCTC emission spectra in the treated plant (see ESI Fig. S1†).

As a second approach, to verify and compare plant uptake of CTC and TC, direct measurements of these antibiotics in pinto bean leaf extracts using LC/MS was performed. Fig. 4 shows the extracted ion chromatograms (*m*/*z* 479 for CTC and *m*/*z* 445 for TC) of the CTC-treated and TC-treated pinto beans. The retention time at 10.12 min observed in CTC-treated plant extract matches that of the CTC standard. The observed splitting of peaks is due to the epimer, 4-epichlortetracycline, which is formed in equilibrium with CTC. Further fragmentation of *m*/*z* 479 produced *m*/*z* 462 ($[M - NH_3]^+$) and *m*/*z* 444 ($[M - (NH_3 + H_2O)]^+$) in the CTC-treated extract. This fragmentation pattern is characteristic of CTC as shown in the MS/MS spectra of the CTC standard. Similarly, the extracted ion chromatogram of TC-treated plant extract showed a distinct peak with retention time at 9.56 min, which matched the retention time of the TC standard. The two peaks in the chromatogram are due to the TC and its epimer, 4-epitetracycline. The MS/MS fragmentation of *m*/*z* 445 in the TC-treated extract also matched that of the TC standard forming the characteristic fragment ions *m*/*z* 427 (loss of NH_3) and *m*/*z* 410 (loss of $NH_3 + H_2O$).

Conclusions

Consistent with our hypothesis, it appears that the related antibiotics TC and CTC are taken up through the roots into the leaves of pinto bean plants. Antibiotic uptake is associated with calcium chelation within the leaves of treated pinto beans, suggesting a possible mechanism of phytotoxicity induced by tetracyclines in pinto bean plants. These results, coupled with our previous findings that protective glutathione *S*-transferases are not induced in these CTC-sensitive plants, begin to elucidate basic biochemical processes that are affected in plants in response to xenobiotic stressors.[12] While it is now well-known that antibiotics are introduced into the environment *via* land application of manure and discharges from sewage treatment plants, studies on antibiotic-induced stress in plants are relatively scarce. It is unlikely that phytotoxic effects will cause complete lethality in plants, but deficient growth and reduced crop production is a demonstrated reality.[11,24] Maximizing crop yields while at the same time reducing environmental impact is an ongoing concern, and characterizing the effects of anthropogenic activity is an essential step in this process.

Traditional methods for cameleon expression are highly effective and will continue to be used for calcium determinations in transgenic organisms. Our strategy of using a biolistic approach provides a highly versatile, straightforward, and rapid alternative to the use of transgenic organisms, allowing for FRET imaging within 2–3 days after transformation. This method has proven to be highly applicable for our environmental analysis of pollutant phytotoxicity and easily adaptable for use in other systems.

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