Identification of Stress in Plants via Femtosecond Laser-Induced Fluorescence and Steady-State Absorption Spectroscopy

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Abstract

We explore the change in optical properties of *Arabidopsis thaliana* in response to four different growth conditions to determine the feasibility of femtosecond laser-induced fluorescence as a method to determine the presence and uptake of uranium. Further, we perform steady-state absorption spectroscopy on extracted cells to better understand how the fluorescence properties, such as excited-state lifetime, are influenced by pigment concentration (chlorophyll A, chlorophyll B, and carotenoids) in response to uranium and drought stress. A positive correlation between the [Chl A]:[Chl B] ratio and the ratio in the lifetimes of the two peaks present in the chlorophyll fluorescence spectrum is identified in most growth conditions. We also observe a discrepancy in this relationship for the uranium exposed plant after 24 hours of exposure, which may be a "shock" response of the plant to the uptake of uranium. We show that femtosecond laser-induced fluorescence of chlorophyll may be a promising method for stress detection in plants and prove suitable for in-field detection of clandestine uranium enrichment activities.

Introduction

There has been recent interest in using plants as biosensors for tracking materials relevant to nuclear proliferation. For example, many studies identified the chemical conditions that enhance the uptake of heavy metals[1-4], including uranium (U)[5-8], for applications such as phytoremediation of pollution and nuclear accidents. Other works investigated uptake efficiency in order to better understand how circulation and transport of toxic materials influence photosynthetic processes. However, further work is necessary to develop methods to monitor this uptake for in-field surveys, especially for uranium contamination.

Interrogation of plants' optical properties provides the most convenient method for in-field detection. Several techniques are commonly employed to study plants' optical properties: pulse-amplitude modulated (PAM) fluorometry[9], hyperspectral imaging and reflectance spectroscopy[10,11], and solar-induced fluorescence (SIF) spectroscopy[12]. PAM fluorometry and SIF use the fluorescence of chlorophyll A (Chl A), which is the main pigment involved in light capture for photosynthesis, to provide information about the photosynthetic chain. In reflectance spectroscopy, the reflection of a broadband light source provides information about the water and pigment content, biomass, and other properties of the plant. The sensing range of PAM fluorometry is limited, so SIF and reflectance spectroscopy are the two most viable methods for remote monitoring. However, the effective utilization of these techniques is hampered by the low signal-to-noise ratio caused by significant environmental solar background.

Femtosecond (fs) laser-induced fluorescence (LIF) is a promising method for standoff optical characterization of plant health. Two main benefits of LIF which make it attractive for remote monitoring of plants' stresses include the ability to efficiently reject slow-varying solar background from rapidly-varying luminescence signal by using a fast-gated detector, and the ability to precisely direct the laser excitation source to a target, narrowing the interrogation region to where plant material is abundant, potentially improving signal-to-noise and analytical capability. While this method is not commonly implemented in the literature for studying plant response to stress, some studies have shown that fs-LIF has been successful in identifying changes in the Chl fluorescence (ChlF) lifetime in response to drought stress in moss[13-15]. Results from previous research indicate that Chl A sites in the photosynthetic chain, which contribute to water uptake and processing, may affect the observed ChlF lifetimes. Work is still needed to identify the information one can retrieve from fs-LIF of plants; however, previous results have shown that fs-LIF can inform about NPQ and monitor disruptions in both PSI and PSII. In this study, we investigate the ChlF spectral shape and lifetime in response to other stressors including uranium contamination.

Chl A can de-excite through three different modes: fluorescence, photosynthesis (i.e. photochemical quenching), and non-photochemical quenching (NPQ) by transferring energy to accessory pigments. There are two main sites along the photosynthetic chain where Chl A resides called photosystems (PS). PSII is the first site, which converts water into H^+ and O_2, generating free electrons. PSII has a reaction center referred to as P680 because Chl A in

PSII fluoresces at 680 nm. The second photosystem, PSI, is involved in using electrons and H^+ to form NADPH from NADP. The reaction center in PSI is referred to as P720 because the Chl A in this site fluoresces at 720 nm. The intensity of the 720 nm peak is typically weaker than the 680 nm peak when measured at room temperature, and generally, PSII is used to determine photosynthetic activity since it is the first step in the photosynthetic chain. Accessory pigments have many functions that assist in photosynthesis and plant function, such as collecting light to transfer to Chl A, receiving excess energy from Chl A, and acting as antioxidants. Bauschmann provides an in-depth discussion of the photosynthetic chain and ChlF[16].

Here, we study *arabidopsis thaliana* grown hydroponically using fs-LIF in order to ascertain the consequences of uranium contamination as a stressor. The steady-state absorption spectra of extracted cells are measured to potentially correlate changes in the pigment content to changes in the fluorescence lifetime. A correlation between the [Chl A]:[Chl B] ratio, total Car content, and ChlF lifetimes is observed. We also identify a distinct signature in U-exposed plants 24 hours post-exposure. This relationship may have implications for stress identification in plants, and we discuss the potential as an in-field method for stress monitoring.

Description of the Work

Hydroponic Growth of Arabidopsis Thaliana

Arabidopsis thaliana seeds are obtained from the Ohio State University Arabidopsis Biological Resource Center (CS72318). Rockwool is the growth medium, and the nutrient solution components can be seen in Table 1. The seeds are planted 1 mm below the surface of the Rockwool and kept in the dark until the sprouts are ~1-5mm above the surface of the Rockwool. Once sprouted, the plants are placed 125 mm below a grow light (390 nm – 730 nm) with a photon flux density of 120 $\mu mol/s \cdot m^2$ and a cycle of 8h on/16h off. Once the roots grow to the bottom of the Rockwool, each plant is transferred to a netted pot and placed in separate containers containing nutrient solution. We grow *a. thaliana* to 50-day maturity before stress exposure. It is important to note that many of the plants have bolted, i.e. grown stems and began to flower, at the time of the stress exposure.

Table 1: Nutrient media concentrations in one gallon of water used for hydroponic growth.

Nutrient	Mass per gallon (g)
KNO_3	0.7
$(NH_4)_3PO_4$	0.1
K_3PO_4	0.35
$MgSO_4$	1.05
H_3BO_3	0.04
Cu-EDTA	0.06
KCl	0.33
Fe-DTPA	0.07
Mn-EDTA	0.25
Zn-EDTA	0.06
Na_2MoO_4	0.004
$Ca(NO_3)_2$	1

Stress Exposure

Previous work demonstrated that uranium uptake is increased when it is in the form of the uranyl ion (UO_2^{2+})[6,8], in solutions with lower pH in the range 5.5-6.5[7], and with minimal concentrations of aqueous phosphorous or calcium[5,17]. In order to optimize conditions for uranium uptake, we prepared a simplified Hoagland nutrient solution following the procedure outlined by Hayek *et al.*[5] Briefly, the solution consists of $MgSO_4$, KCl, NH_4NO_3, $NaHCO_3$, and in some cases $CaCl_2$. Two control solutions are compared; the first contains Ca to compare to drought stress, and the other does not contain Ca to compare with the U stress. These control solutions are used to confirm that any response from the uranium exposure does not solely originate from Ca deficiency. In summary, we expose *a. thaliana* to four different growth conditions: control with Ca, control without Ca, drought (i.e., all water removed from the system), and U (500 µM) stress. The U solution is made by the addition of $UO_2(NO_3)_2$. We record samples at 0-hr and 24-hr of exposure to the new growth conditions.

Cell Extraction

The cells from the leaves of *a. thaliana* are extracted following Method II in Ref [18]. Briefly, three buffer solutions are

made, and the pH of each is controlled with HEPES KOH buffer: grinding buffer, shock buffer, and a storage buffer. Approximately 2 g of leaves are clipped from each plant for each sample, at the same time both days, and are stored in a dark, 4ºC refrigerator for 30 min to allow the sample to dark adapt. The samples are collected from the top of the rosette of leaves, characteristic to *a. thaliana*, to avoid pigment fluctuations that vary from light exposure. The leaves are ground in 1 mL:1 g (vol/w) (ex. 2 g of leaves are ground in 2 mL of grinding buffer) using a mortar and pestle until completely disintegrated. The remaining mixture is then filtered through two layers of Miracloth (pore size ~20 µm) to remove any leftover or unground leaf material. The solution is then transferred to a centrifuge tube that is run for 5 min at 2850 rpm. A pellet forms at the bottom of the tube containing the intact thylakoid membranes. The supernatant is disposed of, and the pellet is then suspended in the grinding buffer for a second time. We run the samples through the centrifuge again at 2850 rpm. Next, the pellet is resuspended in the shock buffer and centrifuged at 4000 rpm for 5 min. After removing the supernatant, the resulting pellet is suspended in the storage buffer and passed through one final cycle of the centrifuge at 4000 rpm for 5 min. This supernatant is disposed of, and the final pellet is then resuspended in the storage buffer and stored in the refrigerator until time for experiments. The centrifuge is refrigerated to a temperature of 4 ºC. The measurements are made within 2 days of sample preparation.

Optical Spectroscopy: fs-LIF and Steady-State Absorption

The experimental setup for fs-LIF is presented in the schematic in Fig. 1. A Ti:sapphire chirped-pulse amplification laser system was used to conduct all fs-LIF measurements. The CPA amplifier is seeded with an 80-MHz pulse train of ~12 fs pulses from a Kerr-lens-modelocked Ti:sapphire oscillator. Compressed output pulses with 1-kHz repetition rate, 130-fs pulse duration (11-nm FWHM bandwidth), a 3.5-mm ($1/e^2$) beam diameter, and 780-nm central wavelength were used for second-harmonic generation (SHG) of the fluorescence pump signal. SHG results in a central laser wavelength of 390 nm using a 200-µm-thick Type I β-$Ba(BO_2)_2$ (BBO) crystal. The pulse

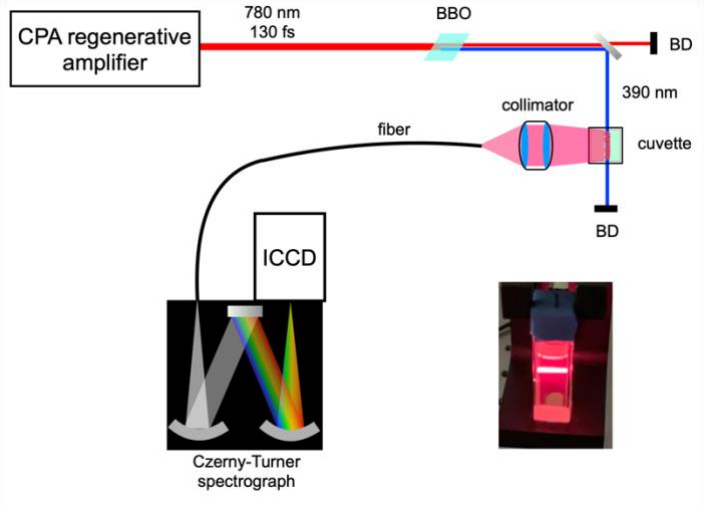

Figure 1: Experimental schematic for fs-LIF of extracted plant cells. The inset shows a real image of the fs-LIF of chlorophyll. BD: beam dump

energy after SHG is 1 µJ, and a dichroic mirror is used to reject any residual 780-nm light. The optimal pulse energy was determined experimentally to prevent optical bleaching of the suspended solution. The fluorescence detection system consisted of a reflective fiber-coupled collimator oriented perpendicular to the beam propagation direction. The collimated signal is coupled into the entrance slit of a Czerny-Turner spectrograph (Andor Shamrock SR303i) used in combination with an intensified charge-coupled device (ICCD, Andor iStar 334T). A 150-l/mm grating blazed at 800 nm is used. Camera triggering was initiated via a 20-Hz TTL signal provided by a digital delay generator (Stanford Research Systems DG535) synchronized to an 8:1-divided RF clock signal from the laser oscillator. The steady-state absorption spectra are recorded with a Varian Cary 50 UV-VIS-NIR spectrophotometer. Pigment concentrations of Chl A, Chl B, and total carotenoids (Car) are calculated following the equations outlined by Porra *et al.* and Lichtenthaler[19,20].

Results & Discussion

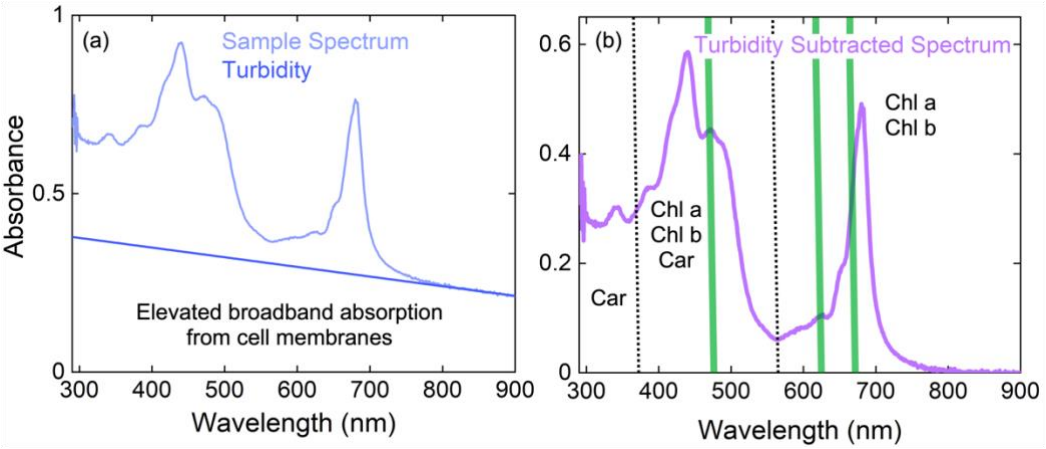

Figure 3: (a) An example absorption spectrum of the extracted thylakoid membranes from *a. thaliana* along with an example turbidity fit; (b) Turbidity-subtracted absorption spectrum, where the regions containing absorption peaks for the pigments of interest are labelled within the dotted lines, and the absorbance in the green shaded regions are used to determine pigment concentration, as outlined in the text.

Figure 2(a) shows an example absorption spectrum of thylakoid membranes extracted from the *a. thaliana* plants. There is an elevated baseline originating from the turbid cell membranes, which can be fitted with a line using calibration points that generally do not contain any relevant absorbing species. We use the region from 800-900 nm and extrapolate to the lower wavelengths. An example linear fit is also shown in Fig. 2(a). Since the absorption solely due to the membrane is not necessarily relevant to the pigment content, which is the main interest of the absorption spectrum, the turbidity can be subtracted, resulting in the spectrum shown in Fig. 2(b).

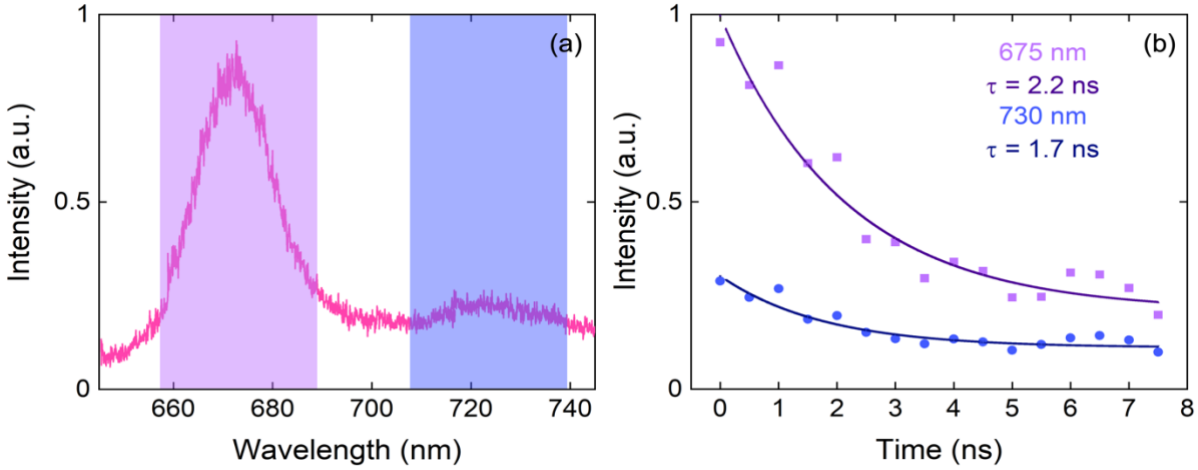

Figure 2: (a) Example chlorophyll fluorescence spectrum recorded for aqueous suspended thylakoid membranes, where the two main peaks of interest are shaded in colors corresponding to the plot in (b). The spectrum is recorded with a 0-ns delay with a 10-ns integration window, and 500 laser shots are averaged. (b) Temporal decay of the main peaks of interest in the ChlF spectrum shown in (a), along with their exponential fits. The initial gate delay is 0 ns. Each spectrum is recorded with a 3-ns gate width in 0.5-ns time steps up to 7.5 ns.

The pigment concentrations and their ratios can provide information about the photosynthetic processes, functional changes in how the plant dissipates collected light, and the response to reactive or radical oxygen species (ROS). The equations derived by Lichtenthaler[19] and Porra *et al*.[20] can be used to determine the concentrations of Chl A, Chl B, and total Car. The main wavelengths that are used are ~665 nm for Chl A, ~640 nm for Chl B, and ~470 nm primarily for the Car[19,20], and these spectral locations are highlighted in green in Fig. 2(b). In general, different carotenoids have similar electronic structures with overlapping spectral signatures, making it difficult to extract concentrations of specific carotenoids which have been studied in detail in plant systems[16]. However, the total carotenoid content, or the relationship of Car to total Chl content, can be a sign of oxidative stress in plants since Car can function as an antioxidant. It is also known that Car can contribute to NPQ of ChlF. Therefore, a change in Car content can mean changes in the activity of NPQ. While NPQ is most commonly measured via PAM fluorometry, it can also be observed on the ps-ns timescale if using fs or shorter pulse durations for excitation of ChlF[13-15,21], and in few cases, has been used to identify signatures of stress in moss[13-15].

Figure 3(a) shows an example ChlF spectrum, and the two peaks corresponding to the two photosystems, PSII and PSI, are at 675 nm and 730 nm, respectively. They are shaded in the respective colors of their temporal decay shown in Fig. 3(b). We take the area under each peak as a function of time and find that the fluorescence decays exponentially and lasts approximately 5-8 ns. The lifetimes extracted from the exponential fits range generally from 1.5-2.5 ns. The lifetime can be dependent on many factors, such as Chl A concentration (triple-state annihilation) and NPQ mechanisms (energy transfer from Chl A to Car). To observe whether one photosystem is affected more than the other, we compare the ratio of the lifetimes for the two peaks.

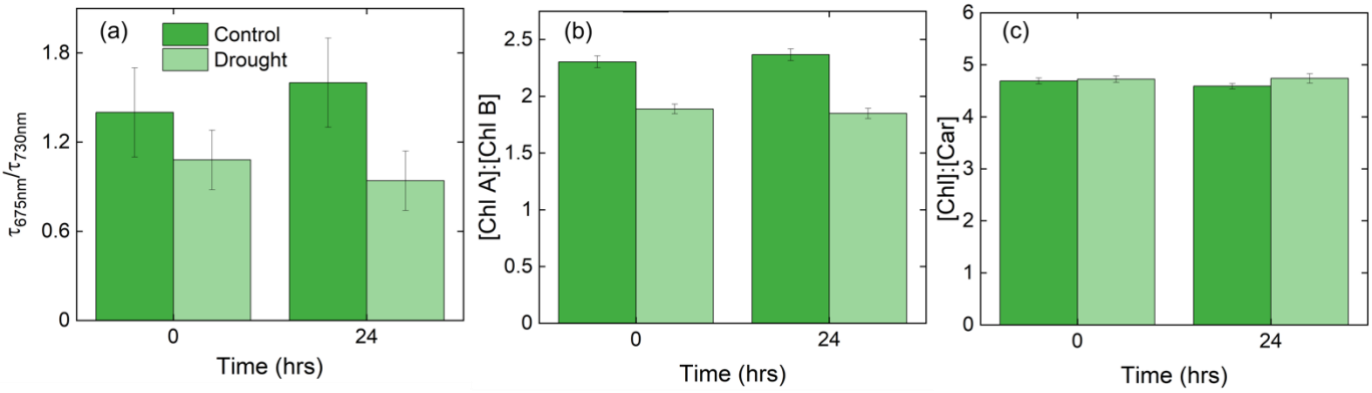

Figure 4: (a) Fluorescence lifetime ratios determined via fs-LIF time-dependent spectroscopy; (b) Concentration of Chl A with respect to Chl B as a function of time of exposure to stress, determined from the steady-state absorption spectrum; (c) Concentration of the total Chl content with respect to total Car content as a function of time of exposure to stress determined from the steady-state absorption spectrum.

First, we compare the scenario of drought to confirm our method in comparison to previous works studying moss stress response to drought with *in-vivo* fs-LIF[13,15]. Prior results show that the lifetime of the peak at 675 nm corresponding to PSII decreases, while that of the peak at 730 nm corresponding to PSI remains unaffected. In order to determine if a similar response is observed in *a. thaliana*, we compare the ratio of the lifetime of the two peaks, shown in Fig. 4(a). We observe that the ratio of the lifetimes does not change significantly for the control; however, there is a decrease in the lifetime ratios observed for drought conditions. A decrease in the ratio corresponds to a decrease in the lifetime of the PSII ChlF with respect to the lifetime of the PSI ChlF. It is well-known that the PSII site is involved in converting water into H^+ and O_2 and producing electrons for the remaining photosynthetic electron transport chain. Therefore, in a drought scenario, the activity of this site will decrease, and be more likely to transfer energy to the accessory pigments.

We then compare the change in [Chl A]:[Chl B], shown in Fig. 4(b), and [Chl]:[Car], shown in Fig. 4(c). We observe a relatively stable [Chl A]:[Chl B] ratio for the drought stress and a simultaneous slight increase in [Chl]:[Car] ratio. While Car are heavily involved in stress remediation through breaking down ROS and alleviating excess energy collection of Chl A, Chl B also contributes to stress remediation. For example, Chl B is an accessory pigment whose absorption spectrum has a similar shape to, but different peak absorption wavelengths from Chl A, and can assist in the light collection in low light scenarios to transfer energy to Chl A to encourage photosynthesis, or to collect more light to minimizes overly excited Chl A which can lead to the formation of ROS. The resulting pigment ratios for drought stress indicate that there is an increase in [Chl B] that is greater than that of [Car]. There also may be a change in structure of carotenoids in response to reduced water content, but not an increase in overall [Car].

Next, we conduct a similar study with uranium exposure by dissolving $UO_2(NO_3)_2$ into the nutrient solution. The results are shown in Fig. 5. The only difference between the control for drought and the control for U exposure is that the nutrient solution does not contain any calcium (Ca), following the procedure described in Ref. [5], which explains that U may follow the uptake pathway of Ca. Therefore, eliminating Ca from the nutrient solution enhances U uptake by the plant species[5].

In Fig. 5(a), a significant decrease in the lifetime ratio for the U stressed plant is observed after 24 hrs of exposure, whereas the lifetime of the control case (no Ca) slightly increases. This trend indicates a rapid “shock” response which occurs in the first 24 hrs of uranium uptake. However, further work is needed to confirm this observation for *a. thaliana* at the same maturity level and U concentration. It is expected to observe increased NPQ of PSII for drought, i.e., decreased lifetime of the peak at 675 nm; therefore, we can assume that there is increased NPQ at 24 hrs of exposure to U. Increased NPQ is common for heavy metal stressed plants[1,4] and can either be attributed to increased carotenoids or other proteins produced as a result of the formation of ROS.

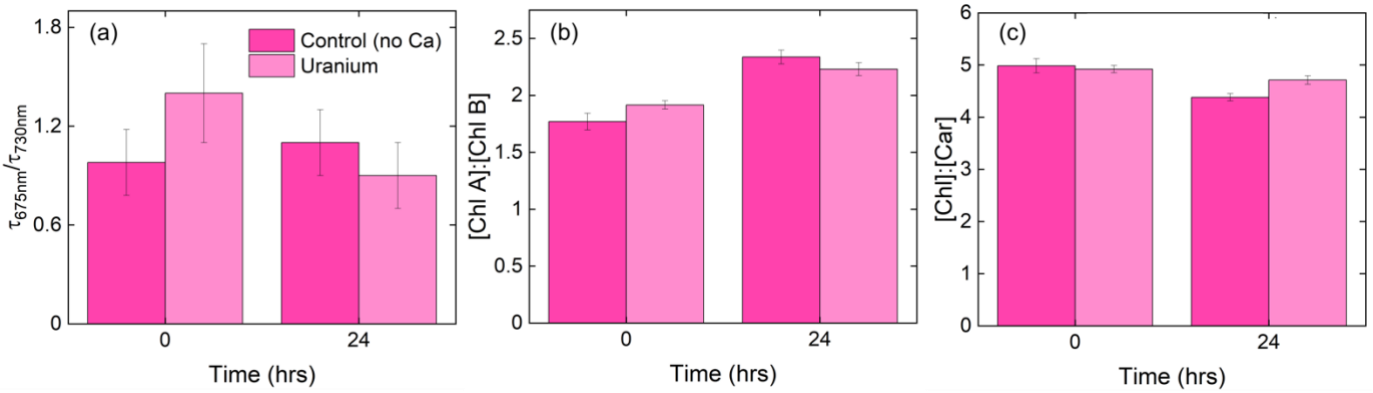

Figure 5: Same structure as Figure 4, except with a Control (no Ca) and uranium stressed a. thaliana.

For the [Chl A]:[Chl B] ratios shown in Fig. 5(b), we observe that both the control (no Ca) and U stressed plants increase from 0 hrs to 24 hrs. In contrast, we see a decrease in [Chl]:[Car] for the control (no Ca) and a relatively consistent ratio over time for the uranium stress. The accompaniment of the increase in [Chl A]:[Chl B] and decrease in [Chl]:[Car] for the control (no Ca) case implies a greater increase in [Car] with respect to the [Chl]. We also see that this corresponds to an increase in the ratio of fluorescence lifetimes. For the drought case in Fig. 4, an increase in [Chl]:[Car] was observed with a simultaneous decrease in the ratio of the lifetimes. Therefore, the change in [Chl A]:[Chl B] appears to be directly proportional to the lifetime ratios of the fluorescence, for natural processes and stresses, like drought.

U stress deviates from this observed trend at 24 hrs, where there is a large decrease in the fluorescence lifetime ratio with a concurrent increase in [Chl A]:[Chl B] and very little change in [Chl]:[Car]. This may have to do with how U influences the photosynthetic chain, where increased NPQ of PSII is observed despite the minimal change in pigment ratios. Although increased NPQ can also be associated with an increase in [Car][1,4], previous work reports increased concentration of other proteins, such as glutathione, can also be a sign of stress associated with NPQ[17,23]. Further work is needed to identify if there is a relationship between glutathione and fs-LIF lifetimes of Chl. A potential explanation for the “shock” response in the *a. thaliana* plant. Previous work found that NPQ decreased upon exposure of 18-day old *a. thaliana* to 50 μM $UO_2(NO_3)_2$[23]. While we do not observe the same effect, the concentration of uranium and age of *a. thaliana* may influence the stress response.

Conclusion

In this work, we investigated the effects of various stressors, such as drought and uranium contamination, on the optical properties of *a. thaliana* using steady-state absorption and fs-LIF spectroscopy. We observe a similar response in the case of drought stress that was observed previously in moss via fs-LIF[13,15], and see that this response may also be correlated to a decrease in [Chl A]:[Chl B] and the corresponding changes in Car content. Furthermore, we observe the same proportional relationship in [Chl A]:[Chl B] ratio to the ChlF lifetime in the control (no Ca) condition, where an increase in [Chl A]:[Chl B] ratio occurs simultaneously with an increase in fluorescence lifetime ratio. For uranium exposed plant samples, we see a significant decrease in lifetime ratio at 24 hours after exposure, which departs from the trend observed with drought or the controls. This suggests a U-induced disruption in the function of Chl A and accessory pigments. Therefore, we have confirmed our measurements using fs-LIF with previous works by observing a similar response to drought stress and use fs-LIF to study U stress in *a. thaliana* for the first time. These results show that fs-LIF has potential as a monitoring method for bio-sensing of nuclear activities, such as uranium enrichment. Further work is needed to confirm this response, and investigate how in-vivo measurements of *a. thaliana* may influence the observed changes in ChlF.

Keywords
Biota; Nuclear Nonproliferation; Laser-Induced Fluorescence; Molecular Dynamics;

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