In vivo diagnostics of early abiotic plant stress response via Raman spectroscopy

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Development of a phenotyping platform capable of noninvasive biochemical sensing could offer researchers, breeders, and producers a tool for precise response detection. In particular, the ability to measure plant stress in vivo responses is becoming increasingly important. In this work, a Raman spectroscopic technique is developed for high-throughput stress phenotyping of plants. We show the early (within 48 h) in vivo detection of plant stress responses. Coleus (Plectranthus scutellarioides) plants were subjected to four common abiotic stress conditions individually: high soil salinity, drought, chilling exposure, and light saturation. Plants were examined poststress induction in vivo, and changes in the concentration levels of the reactive oxygen-scavenging pigments were observed by Raman microscopic and remote spectroscopic systems. The molecular concentration changes were further validated by commonly accepted chemical extraction (destructive) methods. Raman spectroscopy also allows simultaneous interrogation of various pigments in plants. For example, we found a unique negative correlation in concentration levels of anthocyanins and carotenoids, which clearly indicates that plant stress response is fine-tuned to protect against stress-induced damages. This precision spectroscopic technique holds promise for the future development of high-throughput screening for plant phenotyping and the quantification of biologically or commercially relevant molecules, such as antioxidants and pigments.

Raman spectroscopy | plant abiotic stress | carotenoids | anthocyanins

With the global population projected to exceed 9 billion by the year 2050, the task of producing enough food and energy for the world is of utmost importance (1). In anticipation of rising food demand (2), the ability to measure plant stress in vivo is becoming increasingly vital for increasing agricultural production and research. For example, such technologies would allow a farmer to intervene on stress detection and also, make practical the development of crop varieties with increased tolerance to abiotic stress. The field environment requires a comprehensive and rapid screening technology for plant physiological, biochemical, and morphological characteristics (3). Such characteristics can be integrated to predict plant growth potential, biomass processibility, and abiotic stress responses before any visible signs occur in a plant. Plant growth is impacted by unseasonable droughts, cold, increased UV radiation and high-energy blue light associated with atmospheric changes in ozone levels, and fertilizer/irrigation application associated with increased soil salinity (4, 5). Most existing methods for evaluating biochemical characteristics use destructive chemical analyses, which require time and intensive labor. In addition, these methods use strong chemicals, which require special handling and disposal. Currently, in vivo sensing technologies are limited by the time required for detecting a stress response, the types of stress factors that can be detected, the level of stress, and/or physiological changes. For example, reflectance spectroscopy (6), chlorophyll fluorescence spectroscopy (7), IR thermal imaging (8), terahertz time domain spectroscopy (9), and hyperspectral imaging (10) techniques have all been used to measure stress indirectly by focusing on changes in chlorophyll ratios/contents (6, 7), physical changes (9), or water status of plants (8, 10). Surprisingly, Raman spectroscopy has not been widely used. Raman spectroscopy has been used for nondestructive and biochemically specific detection of trace molecules for applications, such as cancer and pathogen detection, agriculture applications, and other plant studies, such as imaging of the plant cell wall (11–15). Near-IR spectroscopy provides a complementary methodology to Raman spectroscopy; however, it has water absorption limitations. The Raman spectroscopic technique, however, is a valuable in vivo tool that deals with highly complex samples in their environment and is relatively insensitive to water. An important advantage of Raman spectroscopy is the ability to interrogate multiple molecular species simultaneously. For the purposes of identifying abiotic stress response in vivo, we address a comparison between molecule biosynthesis and degradation associated with elicited general abiotic stress through utilization of Raman spectroscopy. In this study, two molecules, anthocyanins and carotenoids, were observed across all four abiotic stress factors (Fig. 1). When plants are exposed to abiotic stresses, they undergo highly complex physiological, biochemical, and molecular changes (4, 5, 16). In particular, reactive oxygen species (ROS) accumulate in plants during abiotic stresses, which are highly reactive and toxic, and the plant tries to eliminate them by producing volatile derivatives and antioxidants (17). Carotenoids, which are one of the target molecules in this study, are considered to be the first line of defense against ROS, serving as the main $^1O_2$ quencher in chloroplasts (18–21). The oxidative degradation of accessory photosynthetic pigments, like $\beta$-carotene and other carotenoids, leads to the accumulation of different volatile derivatives, such as...
A simultaneous and in vivo detection of anthocyanins and carotenoids, which are reactive oxygen-scavenging pigments, by the Raman technique.

\[ \beta \text{-cyclocitrinal, which has been shown to serve as a molecular signal responsible for induction of } ^1O_2 \text{-responsive genes (18, 19). Therefore, rapid conversion of } \beta \text{-carotene to } \beta \text{-cyclocitrinal during oxidative stress is suggested to be one of the major defense mechanisms against ROS (18, 19). The second target molecule anthocyanin, a water-soluble pigment derived from flavonoids, has long been associated with plant stress response (16, 22, 23). Anthocyanin protection is twofold: first as an osmotic regulator and second as a light-filtering and free radical-scavenging protective pigment (16). Anthocyanins, which exist almost exclusively as glycosides, can be transported via a plant’s vasculature along with other solutes and eventually accumulate in the cell’s vacuoles. This osmotic regulation through solute concentration protects plants from the damaging effects of various abiotic stresses (16, 22–25). As photo filters, anthocyanins block damaging intense blue, UVA, and possibly, UVB light for the leaf, lowering the light absorption burden for the photosynthetic molecules. In this work, Raman spectroscopy is used for high-throughput stress phenotyping and early stress detection in vivo with improved sensitivity and the ability to interrogate individual molecules, such as carotenoids and anthocyanins, simultaneously.

Materials and Methods

Plant Preparation and Treatment. Coleus lime (Plectranthus scutellarioides) plants were used as an experimental model (23, 26). The seeds were obtained from a commercial source (www.Outsidepride.com). The experiments were carried out in the laboratory with automatic environmental controls (Institute for Quantum Science and Engineering, Texas A&M University). The seeds were initially grown under T5 grow lights on a 16-/8-h light–dark cycle for 11 wk. Next, cuttings were taken from a single fully grown plant to further multiply into cloned plants, because they provided that the plant responses to stress were not caused by genetic discriminations or mutations. These cloned plants were grown under the same conditions mentioned above for 71 d. The experimental model plants were subjected to one of four environmental stresses: salinity, drought, chilling temperatures, or excess light. All plants received a nutrient solution every 2 wk. For the purpose of phenotyping and early stress detection in vivo with improved sensitivity and the ability to interrogate individual molecules, such as carotenoids and anthocyanins, simultaneously.

Chemical Extraction and Analysis. Immediately after spectral data collection, leaves from the replicate plants were sampled for chemical destructive analysis. Square-cut leaf parts from each plant were immediately stored in liquid nitrogen and then a −80 °C freezer. From those frozen samples, eight were used for total carotenoids and five were used for total anthocyanins extraction for each plant. The plant tissues were extracted by the method of Lightenthaler and Buschmann (28) with 100% (vol/vol) acetone. The extracted solution’s absorbance was read at 470, 645, 662, and 750 nm with a Thermo Scientific GENESYS 10 UV-VIS Spectrophotometer. Total carotenoids were calculated using the equations given in ref. 29. Anthocyanins were extracted by using an acidified methanol; 1 μL 50% (vol/vol) methanol, 3% (vol/vol) formic acid, and 47% (vol/vol) distilled water solution was added to each 50 μg plant tissues using the protocol in ref. 22. The extracted solutions were passed through a 0.4-μm filter, and the absorbance was read at 532 nm by the above spectrophotometer as in ref. 22.

Spectroscopic Measurements and Data Processing. A Raman confocal microscopic system equipped with a 532-nm continuous wave (CW) laser was used for the spectroscopic measurements (Horiba; LabRam HR Revolution). Its simplified setup is shown in Fig. 2A. The remote Raman spectroscopic measurements were performed using a custom-built spectroscopic system that is easy to transport to a field. It is considered to be a remote sensing system, because it detects a signal at a 10-cm distance (Fig. 2B). The laser source at range system was a 532-nm CW laser, and the sampling spot size was 200 μm. Plant leaves were placed directly on the sample holder without physical detachment from the plant. Therefore, it is considered as in vivo nondestructive detection. The laser-induced scattered radiation (signal) was efficiently detected by air-cooled CCD cameras. The laser powers were adjusted for the plant tissues without affecting the live cells (0.5 mW with 1 s acquisition time and 10 mW with 10 s acquisition time for microscopic and spectroscopic measurements, respectively). Twenty Raman spectra were collected from four leaves of each plant. These four leaves were selected from different locations of the canopy of the plant. The Raman spectral data of the plants (leaves) were obtained every 12 h during the onset and development of stress until 72 h. Because the leaves are a complex system, we used the mean spectra for additional analysis. The greater contributor of the noise to Raman spectra is the intrinsic fluorescence of molecules in plant tissues. Therefore, to extract Raman signal from the raw spectrum acquired, it is necessary to remove the fluorescence background. The baselines of Raman raw spectral data were corrected by fitting the high-order polynomials with multiple iterations (27). The spectra were further smoothed by the Savitzky–Goïlay algorithm with 15 adjacent points. All data processing programs were written in MATLAB R2013a (The Mathworks).

Fig. 1. A simultaneous and in vivo detection of anthocyanins and carotenoids, which are reactive oxygen-scavenging pigments, by the Raman technique.

Fig. 2. The Raman system setups. (A) Confocal Raman microscopic system. (B) The remote Raman spectroscopic system.
Photosynthetic pigments—anthocyanins and carotenoids—are found naturally in plant tissues. Moreover, anthocyanin biosynthesis is often induced in the leaf’s upper epidermis by excess light irradiation, cold, drought, and saline stresses. Understanding their biosynthesis is, in fact, at the heart of the plant stress tolerance mechanism justifi-
cation (16, 24, 25). By targeting anthocyanins and carotenoids for the pur-
poses of identifying abiotic stress responses in plants, we used a Raman spec-
troscopic technique. To implement Raman spectroscopy, a laser light is used
to excite molecules. The molecules emit light with a new optical frequency
that is downshifted from the incident laser frequency by the amount equal
to their vibrational frequencies. This new color (referred to as Stokes radi-
ation) is further detected with a spectrometer. The Raman spectra of the plants were recorded for 48 h after induction for all four types of stresses (saline, excess light, drought, and cold), including spectra of the unstressed control plants, using both a commercial Raman confocal microscope and a labora-
tory-built (portable) remote Raman system (Fig. 2). The Raman micro-
scopic spectra at 48 h poststress are compared with the unstressed control
plants in Fig. 3. Carotenoids were distinguished in the spectra for the control
plants with distinct narrow peaks at 1,007 and 1,157 cm\(^{-1}\) (30, 31). After abi-
otic stress exposure, the Raman peaks at 539, 623, and 733 cm\(^{-1}\) for antho-
cyanins (32–34) clearly stood out. The set of Raman spectra of the plants was
recorded initially (0 h) and every 12 h for up to 72 h of induction for all four
types of stresses. The explicit height of the Raman peaks changes, indicat-
ing that the concentration of two pigments varies over time. Quantitative
estimations of relative concentration variations of the pigments in plant tis-
sues under stress can be derived from the recorded Raman spectra by using a
least squares regression fitting method. For the sake of simplicity, although
without losing most valuable information, we constructed a fitting as a lin-
ear combination of the recorded Raman spectra of only two pure chemicals:

delicatin chloride (22, 34) and \(\beta\)-carotene (30, 31). A similar least squares
method has been developed (11, 35) for successful diagnostics of breast can-
cer. The obtained fit coefficients represent relative change in the concentra-
tion of the base pigments with certain offset. In fact, these fit coefficients
are functions of both the concentration of particular chemicals and their
Raman scattering cross-sections. Moreover, because of the fact that the plant
tissue is heterogeneous, the fitting coefficients are separately normalized,
which allows the relative change to be quantified. We obtained the relative
changes in carotenoids (brown bars in Fig. 4) and anthocyanin (violet bars in
Fig. 4) as functions of duration of stress. The carotenoids decreased while
the anthocyanins increased the longer the plants were stressed. In the con-
tral plants, carotenoids and anthocyanins levels were not altered. We note
that the Raman spectra of carotenoids (32, 30) and anthocyanins molecules
(32) in live plants have been previously studied one at a time. In this work,
we directly measured the changes in molecule concentrations of anthocyanin
and carotenoid molecules simultaneously. From the plant physiological view-
point, negative correlation between anthocyanins and carotenoids can be
understood as follows. Considering that both of the pigments are involved
in response to ROS, this negative correlation highlights the effectiveness of
the intracellular regulation. Under stress conditions, the strong induction of
ROS (18–21) and the down-regulation of photosynthetic activity lead to the
degradation of carotenoids. Recent research has shown that \(\beta\)-carotene is
rapidly converted to a novel volatile molecular signal \(\beta\)-cyclocitral, which
regulates expression of a set of \(O_{2}\)-responsive genes in plants. Therefore,
it is plausible that the observed reduction of \(\beta\)-carotene in this study can be
explained by its rapid conversion to \(\beta\)-cyclocitral. Although carotenoids
degrade, anthocyanins accumulate as a stress-responding ROS scavenger
(16, 22–25). The strong negative correlation between the two pigments
indicated that signal transduction has fine-tuned the transcriptomic, pro-
tecmic, and metabolic processes to allow the cell to properly adjust to stress
conditions.

**Main Results and Discussion**

**Raman Spectroscopic Detection.** Photosynthetic pigments—anthocyanins
and carotenoids—are found naturally in plant tissues. Moreover, anthocyanin
biosynthesis is often induced in the leaf’s upper epidermis by excess light
irradiation, cold, drought, and saline stresses. Understanding their biosyn-
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indicated that signal transduction has fine-tuned the transcriptomic, pro-
tecmic, and metabolic processes to allow the cell to properly adjust to stress
conditions.

**Remote Raman Spectroscopic Detection of Carotenoids in Plant.** We built a portable at range Raman spectroscopic system. The recorded Raman spectr-
oral changes in carotenoids via a portable Raman spectroscopic platform
were consistent with the Raman microscopic data, thereby showing the capac-
ity of Raman spectroscopy for real life in vivo monitoring of stress responses
of crops in the field (Fig. 5). However, it must be noted that our remote system
was not sensitive enough to measure anthocyanins. Additional improvements
for our system will be to increase the collection efficiency, reduce background
fluorescence, and implement high-sensitivity detectors.
Conclusions

We showed early detection of plant stress responses using in vivo Raman spectroscopic methods, which have improved sensitivity and the ability to interrogate individual stress indicator pigment molecules simultaneously. The variations in the concentration levels of anthocyanins and photosynthetic carotenoids in coleus plants were observed across abiotic stresses, including high salinity, drought, cold, and excess light. These changes over time after stress induction show Raman spectroscopy as a method of accurate measurement of these molecules and are indicative of the functional relationship of these pigments in response to excessive ROS during abiotic stress. This work further understands our current understanding of plant pigmentation by detecting a negative correlation in the levels of anthocyanins and carotenoids during the stress response. The short-term response across multiple abiotic stresses holds promise for a near-ubiquitous method of abiotic stress detection. Finally, our proposed portable system has the capability to become mobile and automated to allow for increased utility in precision agricultural applications for both breeders and commercial producers. The traditional chemical analytical extraction also validated the existence of the concentration changes in either total anthocyanins or carotenoids. In general, the Raman technique could be a cheap, rapid, and nondestructive alternative to chemical analysis. Because it is in vivo, it detects changes of these molecules over time from one plant, which is impossible in destructive chemical analysis.

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