Spectroscopic investigations on plant responses to growth environment

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Spectroscopic Investigations on Plant Responses to Growth Environment

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in fulfilment of the requirement for the degree of
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To my family

献给我的家人

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Abstract

Investigations on plant responses to its growth environment are becoming increasingly important for advanced agriculture. The synergy of plant physiology with optical spectroscopy has enhanced opportunities in these investigations. Systems and protocols have been developed to investigate plant responses to mineral nutrient stress, drought stress and ambient carbon dioxide content variation.

A nutrient stress analyzer was developed for plant mineral nutrient stress investigation. Both a lab-based and a field portable system were developed for acquiring the reflectance spectra from test leaves to provide pre-visual diagnostic information of nutrient deficient conditions. The spectra ranged from 380 to 780 nm were then analyzed for different characteristics such as the Red Edge Inflection Point (REIP), Specific Wavelength Response or the CIELAB color space values. A protocol was established for the systematic use of plant nutrient stress analyzer. The system was then verified for deficiency of calcium (Ca), iron (Fe) and phosphorus (P) status of *Brassica chinensis* L. var *parachinensis* (Bailey) grown under hydroponics conditions. It was found that monitoring of REIP shifts provided a useful spectral cue for pre-visual diagnosis of Ca deficiency in plants. The REIP of progressively maturing control (Ctrl) plants shifted toward longer wavelengths over the experimental period, while that of the Ca-deficient plants remained relatively unchanged. It was determined that if the REIP of a 24-day old plant did not reach or exceed 708 nm, then a Ca-deficient state was indicated. Fe-deprived plants showed rapid entry into a deficient state and the direct effect of Fe on chlorophyll content resulted in statistically significant changes in CIELAB color values two days prior

to the appearance of visible symptoms. The L^* and b^* values were found to be predictive of the plant Fe status that when $L^* > 44$ and $b^* > 22$, plants were in Fe-deficient state. On the other hand, P-deprived plants did not show any spectral changes possibly due to the fact that P content in these plants over the 7-day test period did not fall below the critical nutrient threshold level, and thus the plants did not enter into a P-deficient state. This is the first time a system has been reported to be able to predict plant nutrient stress non-destructively before the appearance of any visible physical symptoms.

A novel idea of using transgenic ‘indicator plants’ concomitantly with a portable spectroscopic system for monitoring plant water status was investigated. *Petunia hybrida* plants transformed with a ‘*rd21A* drought inducible promoter-enhanced green fluorescent protein (EGFP) gene-3’ terminator sequence’ fusion construct via *Agrobacterium*-mediated gene transfer were tested as bioindicators of drought stress. Upon subjection to dehydration, these ‘indicator plants’ were found to emit spectroscopically detectable levels of green fluorescence after 2 hours. The emission peak of EGFP was, however, found to be red-shifted from the expected 509 nm to about 530 nm and this was attributed to the presence of overlapping endogenous autofluorescence from plant cellular components. The temporal and spatial patterns of the EGFP expression characterised by conventional molecular methods were found to correlate well with the patterns obtained by fluorescence spectroscopy.

Finally to monitor plant photosynthesis processes, fiber optic cavity ring down systems were built to detect ambient carbon dioxide gas concentration. The fiber optic cavity was explored as it allowed for a compact all in-one setup. Different resonance cavities, to detect low concentrations of gas with reduced system intrinsic loss, were explored such as

the Fiber Bragg Grating cavity, a passive coupler based cavity, and an active coupler cavity. The active coupler cavity was found to be the best with sufficient ring down times for accurate measurement. This work represents a novel application of fiber optic cavity ring down system for carbon dioxide gas detection.

Keywords: Carbon dioxide, Fiber cavity ring down spectroscopy, Fluorescence spectroscopy, Green fluorescent protein, Plant stress, Reflectance spectroscopy

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A	Round trip loss in percent
a^*	Chroma coordinate in the CIELAB color space
$\mathbf{B}$	PLS regression coefficient-matrix
b^*	Chroma coordinate in the CIELAB color space
$\mathbf{C}$	PLS y-weight-matrix
c	Speed of light
d	Physical separation of the two cavity mirrors
$\mathbf{E}$	PLS x-residual-matrix
$\mathbf{F}$	PLS y-residual-matrix
$\mathbf{G}$	PLS y-residual-matrix
I	Transmitted light intensity
I_0	Incident light intensity
L	Path length of the absorbing medium
L^*	Lightness coordinate in the CIELAB color space
N	Number density of the absorbing species
n	Refractive index
NR^I	Normalized inner reflectance
$\mathbf{P}$	PLS x-loading-matrix
R	Reflectance
R_b	Average reflectance of 380–480 nm
R^E	Eternal reflectance

R^I	Internal reflectance
r^2	Correlation coefficient
S	Line strength
S_r	Relative concentration of the radiant power of the source illuminating the object
T	PLS x-score-matrix
U	PLS y-score-matrix
W^*	PLS x-weight-matrix
X	PLS x-variable-matrix
Y	PLS y-variable-matrix
α	Absorption coefficient
β	Spectral reflectance of the illuminated and viewed object
Γ	Round trip loss in dB
γ	Half linewidth
λ	Wavelength of light
σ	Cross section of the absorbing species
τ	Cavity ring down time
τ_{empty}	Empty Cavity ring down time
t_r	Cavity round trip time
ν	Frequency
$\Delta\nu$	Cavity mode spacing
Δa^*	a^* value difference between sample and control
Δb^*	b^* value difference between sample and control
ΔE^*	Euclidean distance
ΔL^*	L^* value difference between sample and control

List of Symbols

$\Delta\lambda$	Wavelength interval
X, Y, Z	Tristimulus values of the 2° standard observer
X_n, Y_n, Z_n	Tristimulus values of a perfect diffuser or reference white
x_i, y_i, z_i	Color-matching functions of CIE standard colorimetric system.

Chapter 1 Introduction

1.1 Background

The challenge to maintain sufficient food supply to feed an ever-increasing population has intensified in the face of loss of arable land and dwindling natural resources. As such, traditional farming methods based primarily on a regimented program of chemical and natural inputs applied across open fields are no longer sustainable agricultural approaches. Traditional farming methods often result in non-optimal resource distribution, affecting crop yield, quality and profit margin. In addition, poor management of resource utilization has resulted in negative ecological consequences on soil physio-chemical properties, biodiversity, groundwater quality and the environment, with adverse implications for long-term agricultural sustainability (Bajwa 2002). Consequently, there is strong impetus to develop turnkey technologies that allow for maximal crop productivity and quality, while maintaining ecological integrity through precise and site-specific application of chemical and natural resources (Griepentrog and Blackmore 2004). A rapidly advancing strategy is to use information technology in the domains of remote sensing, global positioning and geographical informational systems to track crop status and then linking this information with resource management for more targeted use of inputs (Bajwa 2002). Such ‘needs-based’ application of inputs reduces environmental loading and promotes natural resource conservation. From the foregoing, it is logical then that enhancing deeper understanding of plant responses to its growth environment would form the foundation for developing sustainable agricultural practices.

Plant responses to the environment include the responses to available mineral nutrients, water stress, heat stress, environmental air pollution and so forth. The key factors that have a significant impact on plant growth and final crop yield are availability of nutrients and water as well as ambient CO₂ levels via their effect on plant photosynthetic efficiency. Hence, the development of spectroscopic systems for monitoring plant responses to nutrient deficiency, water stress and measurement of ambient CO₂ levels form the basis of investigations described in this thesis.

Plant nutrition is very important for plant survival and growth. Lack of essential nutrients will seriously affect the growth of the plant. Therefore nutrient management is a major problem for farmers and agricultural dealers besides environment and pests. There are many ways to detect plant nutrient content and these can be broadly divided into two groups: plant analysis and tissue testing.

Plant analysis is the determination of the total elemental content, made by extraction from oven-dried, ground plant tissue. In plant analysis laboratories, a number of different analytical procedures are required to assay for different essential elements:

- Kjeldahl digestion (Nelson and Sommers 1980) or dry combustion (Jones, Wolf et al. 1991) for nitrogen (N)
- Colorimetry for phosphorus (P)
- Flame atomic absorption spectrometry (Watson 1998) for calcium (Ca), iron (Fe)

A tissue test is conducted on an extracted sap in the field rather than collecting tissue that will be sent to a laboratory for analysis. In general, tissue testing is conducted using chemically treated papers or test strips, test tubes or vials, and specially prepared reagents.

The development of a color and its intensity are used as indicators of the presence of an element (normally its ion) and its concentration, respectively. Kits such as the HACH kits are available and can be used to conduct such tests (Scaife and Stevens 1983). There are new instruments that can be used, like the nitrate meter, for determination of the nitrate anion (Stone, Solie et al. 1996; Sui, Wilkerson et al. 1998). A more recent development has been the use of chlorophyll meter for the assessment of nitrogen status of plants (Blackmer and Schepers 1994; Piekielek and Fox 1992; Piekielek, Fox et al. 1995; Schepers, Francis et al. 1992; Smeal and Zhang 1994; Tracy, Hefner et al. 1992).

There are limitations to the above methods. Plant analysis is time consuming as it needs sample preparation and only trained professionals can do the analysis. In tissue testing, test kits are subjective. For example, when dealing with a suspected nutrient element deficiency, the sampling time depends on visual symptom seen by naked eye. Furthermore, nitrate meter only measures nitrate anion. Finally all the above methods need destructive sampling. The chlorophyll meter allows for non-destructive sampling but it only measures the leaf greenness, and then relates the leaf greenness with nitrogen (N) status in plant. But oftentimes it may not be nitrogen causing the change of the leaf greenness.

Much research on remote sensing (Curran 1989; Doerffer 1988; Gitelson and Merzlyak 1996, 1997; Kondratyev and Pozdnyakov 1987; Lichtenthaler 1988; Pillai, Tian et al. 1998; Zagolski, Pinel et al. 1996), which includes using image processing, global positioning system, distributed control system (like controller area networks), and so on, has been done for nutrient management. Remote sensing technology provides the

opportunity for detecting large fields. But it suffers from the background noise of ground and shadows, and it needs to compensate for ambient illumination variations.

Therefore nutrient stress detection system, which is portable (can be used in field), non-destructive (no sample preparation), user friendly (not only can be used by professionals), with early detection capability and objective, has to be developed.

Plants have direct relation with various kinds of gas in atmosphere, CO₂, O₂ (photosynthesis) (Keutgen and Chen 2001; Larbi, Abadía et al. 2004; Pal, Karthikeyapandian et al. 2004; Singh and Blanke 2000), volatile organic compounds like ethylene and methane (plant mature or fruit stage) (Guenther, Zimmerman et al. 1994; Lu, Wassmann et al. 1999). Among all these, CO₂ is the one that has been paid more attention because of the overwhelming greenhouse effect.

Since the industrial revolution and expansion of agriculture around 200 years ago, we have been pumping additional carbon dioxide into the atmosphere. Today the concentration of carbon dioxide is around 375 ppm, which is approximately 30% greater than in the 18th century (Murray 1997). Plants absorb CO₂ during photosynthesis, store carbon in roots, stems, leaves, and the soil. If plants are subjected to mineral nutrient stress or are grown in elevated CO₂ concentration conditions, the photosynthesis rate or respiration rate might be changed, therefore, less or more CO₂ gas will be absorbed, and further stress might occurred (Hamer 2003; Keutgen and Chen 2001; Larbi, Abadía et al. 2004; Pal, Karthikeyapandian et al. 2004; Pieters, Paul et al. 2001; Singh and Blanke 2000). Researchers have been working on the relation of plants and CO₂ for decades (Larbi, Abadía et al. 2004; Manderscheid, Bender et al. 1995; Pal, Karthikeyapandian et

al. 2004; Sasek and Strain 1991), and several systems have been developed to detect CO₂ gas, e.g. photosynthetic measuring system (LI-6400, LI-COR, Inc., USA), CO₂ infrared gas analyzer (PP Systems, UK). These systems use expensive mid-IR laser sources and can only perform localized measurement. Therefore, a system, which is highly sensitive (the CO₂ concentration in ambient air is in the level of hundreds of ppm), capable of remote sensing and low cost is preferred.

1.2 Objectives and Scope

The main goal of this project is to design and develop optical systems based on spectroscopy with associated spectral analysis to study and measure plant responses to its growth environment. Different environmental factors cause different responses that require both morphological and physiological studies. This multi-disciplinary study involving plant physiology, optical spectroscopy and pattern analysis will be used to develop a protocol for nutrient stress analysis.

The scope of this research includes:

1. Develop a nutrient stress analyzer for plant mineral nutrient stress detection.

To develop such an analyzer, the responses of plant to mineral nutrient stress have to be understood, so that suitable parameters can be enhanced and measured. The nutrient stress analyzer will be developed, based on morphological studies of plant responses measured using reflectance spectroscopy and analyzed using spectral strategies. The analyzer has to be portable, remotely accessible, non-destructive, user friendly, and must be able to provide early detection capability and an objective assessment of the physiological state of the plant. The nutrient stress analyzer will be

tested for detection of deficiency states of three essential nutrient elements and a protocol will be established for plant stress detection.

2. Develop transgenic 'indicator plants' and a portable system for drought stress detection.

Transgenic 'indicator plants' that fluoresce upon entry into water stress will be developed by insertion of a novel gene construct comprising a drought-inducible promoter linked to the enhanced green fluorescent protein marker gene into *Petunia* plants. The interrogation of drought-induced fluorescence emissions using a fiber optic based spectroscopic system modified from that designed for nutrient stress analysis is employed. The response of the 'indicator plants' to water stress will be characterized using fluorescence microscopy and spectral analysis and the pattern of drought-induced green fluorescence emissions will be compared with conventional molecular analyses of EGFP marker gene expression.

3. Development of a new fiber optic cavity ring down spectroscopy for CO₂ gas detection to study plant response to ambient changes.

A fiber optic cavity ring down spectroscopic system will be developed for ambient CO₂ gas detection using all fiber designs. Different system configurations will be explored and tested to achieve high sensitivity and low intrinsic loss. This is the first report describing the use of a fiber optic cavity ring down system for carbon dioxide gas detection in the weak absorption near infra-red region.

1.3 Organization of Thesis

This thesis contains seven chapters:

In chapter one, the background and objectives were presented. It emphasizes the need for inter-disciplinary approaches in this project.

In chapter two, a literature review covering the theories behind various methods used in this project was presented. These theories cover key aspects of plant physiology, reflectance spectroscopy, fluorescence spectroscopy and cavity ring down spectroscopy.

In chapter three, a nutrient stress analyzer was developed for plant mineral nutrient stress detection. Both hardware and software development were described. Strategies used for data analysis were presented in detail.

In chapter four, trial tests of the nutrient stress analyzer were performed on calcium-, iron- and phosphorus-stressed plants. Different threshold criteria were set for distinguishing the respective nutrient stresses in the plants.

In chapter five, 'indicator plants' carrying the enhanced green fluorescent protein (EGFP) marker gene were generated for concomitant use with a portable spectroscopic system for drought stress detection under laboratory conditions. The temporal and spatial patterns of the EGFP expression were analyzed by both conventional gene expression characterization and fluorescence spectroscopy.

In chapter six, fiber cavity ring down spectroscopy was explored for CO₂ gas detection. Several configurations—fiber bragg grating (FBG) cavity, passive coupler fiber cavity and active coupler fiber cavity, were set up and tested.

In chapter seven, the conclusions and recommendation for future work are addressed.

In the appendix, a detailed protocol for the nutrient stress analyzer spectrum acquisition is provided.

Chapter 2 Literature Review

This chapter will briefly review the theories behind various spectroscopic methods used in this project. These theories cover the plant physiology, reflectance spectroscopy, fluorescence spectroscopy and cavity ring down spectroscopy. An understanding of these multi-disciplinary topics is essential to comprehend and detect the response of plants to environment.

2.1 Plant Physiology

2.1.1 Plant Leaf

Leaf of a plant is a primary photosynthesizing organ with photosynthesis occurring in chloroplasts where chlorophyll pigments locate (Marshall and Roberts 2000). The shape and form of the leaf vary widely from species to species. A typical plant leaf is shown in Figure 2.1. Leaf has two main parts, the blade and the petiole. The blade contains two types of tissues: the parenchyma in which most of the photosynthetic activity takes place, and the vessels (or veins) through which pass the rising and descending sap. The petiole joins the leaf blade to the stem. It contains both forms of conductive tissue connected to similar tissues in the leaf blade and in the stem, and thick-walled cell tissue, which provide rigidity. Leaves have limited growth pattern: when they reach maturity they do not grow further. There are differences in composition between the young leaves that have recently emerged from the bud, mature leaves and senescent leaves.

Figure 2.1 A typical plant leaf.
(<http://conference.uhh.hawaii.edu/hsta/Page7leaf.gif>)

The cross-section of a typical dicotyledonous plant leaf is showed in Figure 2.2. It generally consists of upper epidermises, lower epidermises, and mesophyll in the middle leaf (Hopkins 1999). The mesophyll includes the major photosynthetic tissues: palisade parenchyma cells and the spongy parenchyma cells. The chloroplasts are readily seen located along the walls of the parenchyma cells. Within the chloroplast are long slender strands called grana within which the chlorophyll are located. The granum is 500nm in length and 50nm in diameter. Clearly, this is of the dimension of the wavelength of light and produces considerable scattering of light entering the chloroplasts. The chloroplasts are generally more abundant on the upper side of the leaf in the palisade cells and hence account for the darker appearance of the upper leaf surface compared with the lower surface. Pigments generally found in chloroplasts are chlorophyll—green color (65%), carotenes—orange or red-orange color (6%) and xanthophylls—yellow color (29%). Carotenes and xanthophylls belong to carotenoid pigment. As the leaf ages, the chlorophyll pigment is degraded and the more stable carotenoid pigments account for the brilliant orange and yellow colors.

Figure 2.2 Internal structure of a typical dicotyledonous leaf.
(http://www.lions.odu.edu/~kkilburn/bio109/plant_leaves.htm)

2.1.2 Plant Nutrition

Plant nutrition can be defined as the totality of the relationships which plant has with chemical elements and compounds, and which occur both within the plant and at its interfaces with external environment (Martin-prével, Gagnard et al. 1987). These relationships can be grouped according to their nature and purpose into: water requirements, carbon, and mineral nutrition (nutrients other than C, H and O). Sixteen essential elements are required by plants. Table 2.1 shows where the essential elements come from and their functions to plants (Atwell, Kriedemann et al. 1999).

Table 2.1 Function and primary source of essential plant nutrients.

Elements	Main Function In Plants	Primary Sources
Boron (B)	Enzyme synthesis; cell division; sugar transport	Growing media
Carbon (C)	Part of all organic compounds	CO ₂ in air
Calcium (Ca)	Structural component of cell walls; cell elongation	Growing media
Chlorine (Cl)	Photochemical reaction in photosynthesis	Rainwater
Copper (Cu)	Enzyme activator; nitrate reduction; respiration	Growing media
Iron (Fe)	Chlorophyll synthesis ; oxidation reduction reaction; enzyme activator	Growing media
Hydrogen (H)	Forms main structural components	Water
Potassium (K)	Protein synthesis; translocation of carbohydrates; enzyme activation; universal cation	Growing media
Magnesium (Mg)	Components of chlorophyll, enzyme activator, metabolism	Growing media
Manganese (Mn)	Oxidation-reduction reactions; nitrate reduction; enzyme activator	Growing media
Molybdenum (Mo)	Nitrate reduction; fixation of atmospheric nitrogen by legumes	Growing media
Nitrogen (N)	Components of proteins, chlorophyll, nucleic acids	Growing media
Oxygen (O)	Forms main structural components	Water, air
Phosphorus (P)	Energy transfer; metabolism, nucleic acids, phospholipids	Growing media
Sulfur (S)	Constituent of proteins; involved in respiration and nodule formation	Growing media
Zinc (Zn)	Enzyme activator, regulates pH of cell sap	Growing media

These elements can be classified as macronutrients and micronutrients (Table 2.2). Macronutrients are required in large quantities while micronutrients are needed in smaller quantities.

Table 2.2 Macronutrient and micronutrient (Atwell, Kriedemann et al. 1999).

Macronutrient	C	Ca	H	K	Mg	N	O	P	S
Micronutrient	B	Cl	Cu	Fe	Mn	Mo	Zn		

Mineral nutrition can also be separated into two groups, mobile and immobile nutrients, as shown in Table 2.3. Mobile elements mean that the elements can be transferred from older leaves to younger growing part of the plant. In the period of growth, if the plant is deficient of a mobile element, the element will move from the old leaves to the young leaves to ensure continuing plant growth. Deficiency symptoms should then become evident first in the old leaves. Immobile elements fix and stay where they are incorporated into the structure of the plant or its metabolites. The deficiency symptoms of immobile element appear first in the young leaves, because the metabolic rate growth of the young leaves is higher than older leaves. Thus young leaves need a larger supply of the nutrient element, and exhaust the available nutrient element faster.

Table 2.3 Mobility of plant mineral nutrient (Atwell, Kriedemann et al. 1999).

Immobile	Ca	Fe	B	Cu	Mn	S
Mobile	N	P	Mg	K	Zn	

Plant may suffer hunger as a result of various causes (Deturk 1941), among which are: insufficient supply in the growing media; too low a degree or rate of solubility; physiological unavailability, i.e. inhibition of absorption by other ions or by unfavorable hydrogen-ion concentration; physiological unavailability within the plant, i.e., inability of the plant to utilize an element after absorption. According to Figure 2.3 (Munson and Nelson 1990), if the concentration of an element is lower than the critical value, the plant will be in the state of “**hidden hunger**” (visual symptoms can not be observed with naked eyes), thereafter gradually moving to “**visual deficiency**” state (deficiency symptoms can be seen with naked eyes). Different nutrient elements have different critical values. The critical value is defined as the concentration below which yields decrease or deficiency symptoms appear. Because the exact concentration of a nutrient below which yields decline is difficult to determine precisely, some define the critical level as the nutrient concentration at 90–95% of maximum yield.

Figure 2.3 Relationship between plant growth and nutrient concentration.

2.2 Spectroscopy

Light is electromagnetic radiation. It is emitted or absorbed when electrons of an atom change their energy states. A photon is emitted when an electron “jumps” from a higher energy state to a lower energy state. The photon must somehow get rid of that energy and it does so by emitting energy in the form of electromagnetic radiation. The electromagnetic spectrum is shown in Figure 2.4.

Spectroscopy is the study of the interaction between light and matter. As photons hit the sample surface, some are reflected, some pass through the sample, and some are absorbed by the sample. In this section, various kinds of spectroscopy based on reflectance and absorption were described. These are the base theories for the experiments described in following chapters.

Figure 2.4 Electromagnetic spectrum from gamma rays to radio waves.
(<http://www.crisp.nus.edu.sg/~research/tutorial/em.htm>)

2.2.1 Reflectance Spectroscopy

2.2.1.1 Background

Reflectance spectroscopy is an extension, via instrumentation, of human vision. Human eye is a crude reflectance spectrometer: we look at a surface and see color. Our eyes and brain are processing the wavelength-dependent scattering of visible-light photons to reveal something about what we are observing, like the red color of hematite or the green color of olivine. A modern spectrometer, however, can measure finer details over a broader wavelength range and with greater precision.

Reflectance spectroscopy is the study of light as a function of wavelength that has been reflected or scattered from a solid, liquid, or gas. The reflectance occurs not only at the surface, but from the interior as well. Photons incident on a surface are absorbed, reflected, refracted or diffracted at the various interfaces inside the material. In reflectance spectroscopy, photons that are reflected from both surface and interior are detected and measured. Light reflected by scattering media generally consists of both diffuse and specular components. The diffuse component arises through multiple scattering by particles near the surface but inside the material. However the specular component is determined by the material surface property (Birth and Hecht 1990).

Most spectral measurements of plant leaves previously reported were made over a waveband from 500–2500nm (Bargen, Meyer et al. 1992). There are measurements for predicting nitrogen content and concentration from various leaf reflectance spectra: corn leaves (Daughtry, Walthall et al. 2000; Lee and Searcy 2000; McMurtrey, Chappelle et al. 1994; Yoder and Pettigrew-Crosby 1995), maple leaves (Yoder and Pettigrew-Crosby 1995), and sunflower leaves (Penuelas, Gamon et al. 1994). Some measurements

concentrate on the characteristics in the far-red region of reflectance spectra of leaves with various chlorophyll content: maple leaves (Vogelmann, Rock et al. 1993), wheat leaves (Munden, Curran et al. 1994), and slash pine (Cibula and Carter 1992). Carter et al. (Carter 1993, 1994; Carter and Miller 1994) tried to find the ratios of leaf reflectance in narrow wavebands that can be taken as indicators of plant stress and the effects of water content on the leaf reflectance spectra (Carter 1991), but none of their findings could be used to predict plant stress or discriminate different stresses. Ahern (Ahern 1988) used the leaf reflectance spectra to detect the effects of bark beetle of lodgepole pine. Jacquemoud et al. (Jacquemoud, Verdebout et al. 1995; Jacquemoud, Baret et al. 1995; Jacquemoud and Grossman 1996; Jacquemoud and Fourty 1996; Jacquemoud, Ustin et al. 1996) tried to extract the leaf biochemistry information from leaf reflectance data, but the results were not reliable except for leaf water content. Some have also used the leaf reflectance data to discriminate crop and weed in field (Borregaard, Nielsen et al. 2000). Other than focus on specific wavelengths, looking at the whole reflectance spectrum might be able to pick up more information. With the help of color system, checking on hundreds of or even thousands of wavelengths from the whole spectrum is not necessary.

2.2.1.2 Color System

Starting from the nineteenth-century experiments of Maxwell, Young and Helmholtz in mixing colored lights, by the late 1920s, the eye's sensitivity to light relative to wavelength was established, the so-called 'standard observer' defined and the Commission Internationale de l'Eclairage (CIE) system of color measurement constructed (Thompson 1995). Since then, considerable improvements have been incorporated into the system. With the development of computer, complex color measurements and calculations are now routinely used for industrial process such as paint formulation, color

match prediction, and control of the appearance of dyed textiles, and in food industry for ingredient standardization and product quality control (Mcdonald 1997).

The CIE system of color measurement transforms the reflection or transmission spectrum of the object into three-dimensional space using the spectral power distribution of the illuminant and the color-matching functions of the standard observer. The CIE 1976 (L^* , a^* , b^*) color space, know as CIELAB color space, provides a three-dimensional representation for the perception of color stimuli (Berger-Schunn 1994; Wyszecki and Stiles 2000). Figure 2.5 shows the three-dimensional CIELAB color space.

Figure 2.5 Three-dimensional CIELAB color space.

If two points in the color space, representing two stimuli, are coincident then the color difference between the two stimuli is zero. As the distance in the color space between two points increases, it is reasonable to assume that the perceived color difference between the stimuli that the two points represent increases accordingly. One measure of the difference in color between two stimuli is the Euclidean distance, ΔE^* between the two points in the three-dimensional color space, given as (Wyszecki and Stiles 2000):

$$\Delta E^* = \left[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2 \right]^{1/2} \quad (2-1)$$

where ΔL^* , Δa^* , Δb^* are the L^* , a^* , b^* value difference between sample and control, given as (Wyszecki and Stiles 2000):

$$\Delta L^* = L_s^* - L_c^*, \text{ positive—lighter; negative—darker.} \quad (2-2)$$

$$\Delta a^* = a_s^* - a_c^*, \text{ positive—redder; negative—greener.} \quad (2-3)$$

$$\Delta b^* = b_s^* - b_c^*, \text{ positive—yellowier; negative—bluer.} \quad (2-4)$$

where L^* is lightness coordinate, a^* and b^* are the chroma coordinates in the CIELAB color space (subscript s =sample, c =control), given as (Wyszecki and Stiles 2000):

$$L^* = 116 \times (Y/Y_n)^{1/3} - 16 \quad \text{for } Y/Y_n > 0.008856; \quad (2-5)$$

$$L^* = 903.3 \times Y/Y_n \quad \text{for } Y/Y_n \leq 0.008856; \quad (2-6)$$

$$a^* = 500 \times (f(X/X_n) - f(Y/Y_n)) \quad (2-7)$$

$$b^* = 500 \times (f(Y/Y_n) - f(Z/Z_n)) \quad (2-8)$$

where $t = X/X_n$, Y/Y_n , Z/Z_n , $f(t) = t^{1/3}$, for $t > 0.008856$; $f(t) = 7.787 \times t + 16/116$, for $t \leq 0.008856$. X , Y , and Z are the tristimulus values of the 2° standard observer; X_n , Y_n and Z_n are the tristimulus values of a perfect diffuser or reference white, given as (Wyszecki and Stiles 2000):

$$X = k \sum_{\lambda=\lambda_a}^{\lambda_b} (\beta(\lambda) \cdot S_r(\lambda) \cdot x_i(\lambda) \cdot \Delta\lambda) \quad (2-9)$$

$$Y = k \sum_{\lambda=\lambda_a}^{\lambda_b} (\beta(\lambda) \cdot S_r(\lambda) \cdot y_i(\lambda) \cdot \Delta\lambda) \quad (2-10)$$

$$Z = k \sum_{\lambda=\lambda_a}^{\lambda_b} (\beta(\lambda) \cdot S_r(\lambda) \cdot z_i(\lambda) \cdot \Delta\lambda) \quad (2-11)$$

$$k = \frac{100}{\sum_{\lambda=\lambda_a}^{\lambda_b} (S_r(\lambda) \cdot y_i(\lambda) \cdot \Delta\lambda)} \quad (2-12)$$

where $\beta(\lambda)$ is the spectral reflectance of the illuminated and viewed object; $S_r(\lambda)$ is the relative concentration of the radiant power of the source illuminating the object; $\Delta\lambda$ is wavelength interval; $x_i(\lambda)$, $y_i(\lambda)$ and $z_i(\lambda)$ are color-matching functions of CIE standard colorimetric system.

2.2.2 Fluorescence Spectroscopy

2.2.2.1 Background

Fluorescence is the property of some atoms and molecules to absorb light at particular wavelengths and to subsequently emit light of longer wavelengths after a brief interval. As the theoretical underpinning of fluorescence became more and more understood, and the rapid development of instrumentation and software, fluorescence has proven to be a versatile tool for a myriad of applications. It's a powerful technique for studying molecular interactions in analytical chemistry, biochemistry, cell biology, physiology, nephrology, cardiology, photochemistry, and environmental science (Mycek and Pogue 2003; Rigler and Elson 2001).

Fluorescence emission from plants is a well-known phenomenon. Fluorescence is useful in the study of photosynthesis (Chappelle, McMurtrey III et al. 1991; Rosema, Verhoef et al. 1991) and it also has been used to monitor plant stress (Günther, Dahn et al. 1994; Morales, Belkhodja et al. 1999; Valentini, Cecchi et al. 1994). The benefits of plant fluorescence measurement though have yet to be applied in a commercial production

setting. There are two main reasons that basically relegate fluorescence measurements to the laboratory: 1) the need for a dark environment and 2) the need for "dark adaptation" of the plant tissue prior to testing. One present development, which overcomes these limitations, is the use of the Green Fluorescent protein (GFP).

2.2.2.2 Green Fluorescent protein

2.2.2.2.1 Working Principle

Green Fluorescent protein (GFP) is a naturally fluorescent protein isolated from the jellyfish *Aequorea Victoria* and other marine organisms. It converts the blue chemiluminescence of other proteins, aequorin or luciferase, into green fluorescent light (Morin and Hastings 1971; Ward 1979), presumably to reduce scattering and hence improve penetration of the light over longer distances. The molecular cloning of GFP cDNA from *A. Victoria* (Prasher, Eckenrode et al. 1992) and the demonstration by Chalfie, Tu et al. (1994) that this GFP can be functionally expressed in bacteria and nematodes have opened exciting new ways to investigation in cell, developmental, and molecular biology (Chalfie 1995; Heim and Tsien 1996).

One of the great advantages of GFP as a reporter of gene expression is its high level of stability. This stability appears to be the consequence of its unique three-dimensional structure (Ormö, Cubitt et al. 1996; Wu, Liu et al. 1997; Yang, Moss et al. 1996, 1997), as shown in Figure 2.6.

Figure 2.6 Structure of GFP.

(http://www-jackson.ch.cam.ac.uk/Jackson_research_files/gfp3.jpg)

To understand the working principle of GFP as reporter gene, information of gene cloning is needed. Figure 2.7 shows the basic steps of gene cloning (Brown 1995). A fragment of DNA, containing the gene to be cloned (e.g. GFP gene), is inserted into a circular DNA molecule called 'vector', to produce a chimera (recombinant DNA molecule). The vector acts as vehicle that transports the gene into a host cell, which is usually a bacterium. Within the host cell, the vector multiplies, producing numerous identical copies of itself and the gene that it carries. When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication take place. After a large number of cell divisions, a clone of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule.

Figure 2.7 Basic steps in gene cloning.

2.2.2.2.2 Factors affect detection of GFP

Several parameters affect the detection of GFP. These parameters include the type of GFP used (mutant versus wild-type forms), the concentration of GFP, the effects of background autofluorescence, and the time required for posttranslational modifications to form GFP's chromophore.

Other factors, such as mRNA stability and factors regulating translation, or the stability of fusion proteins, may also contribute to achieving critical levels of GFP. In cases where fusions are made with other proteins that are tightly localized within the cell, high local

concentrations of the fusion protein may play an important role in the ability to detect GFP fluorescence.

GFP types

There are many limitations to the use of wild-type GFP:

First, the excitation wavelengths are not optimal for use with common filter sets. Figure 2.8 shows the excitation and emission spectra of wild type GFP. Excitation near the major peak (i.e., 395 nm) causes photoisomerization (Cubitt, Heim et al. 1995) and significant autofluorescence when used *in vivo*. Although the 470 nm excitation peak is subject to less photoisomerization and autofluorescence, its much lower extinction coefficient causes the fluorescence light to be very weak. Second, the chromophore in the wild-type GFP forms rather slowly, potentially limiting its use to detect fast transcriptional events (Heim, Prasher et al. 1994; Heim, Cubitt et al. 1995).

Figure 2.8 Excitation (dark) and emission (gray) spectra of wild type GFP.

Recently, isolated mutant versions of GFP affect the excitation or emission properties of the protein, making it either a more brightly fluorescent protein, or altering the wavelengths of maximum excitation or emission. Figure 2.9 shows the excitation and emission spectra of five mutant GFPs (Enhanced blue fluorescent protein (EBFP), enhanced cyan fluorescent protein (ECFP), enhanced green fluorescent protein (EGFP), enhanced yellow fluorescent protein (EYFP), red fluorescent protein (DsRed)). The according peak wavelength positions are shown in Table 2.4. The additional colors of mutant GFP would also be valuable to permit multicolor comparison of two or more protein localizations or gene expression levels, as well as to enable fluorescence resonance energy transfer (Cubitt, Heim et al. 1995; Heim, Prasher et al. 1994; Heim and Tsien 1996; Rizzuto, Brini et al. 1996; Yang, Kain et al. 1996).

Figure 2.9 (a) Excitation and (b) emission spectra of different types of mutant GFPs. (http://www.plantsci.cam.ac.uk/Haseloff/imaging/LeicaSP/fluorescent_spectra.html)

Table 2.4 Excitation and emission peak wavelength of different mutant GFPs.
(http://www.plantsci.cam.ac.uk/Haseloff/imaging/LeicaSP/fluorescent_spectra.html)

Fluorochrome	Excitation peak (nm)	Emission peak (nm)
EBFP	380	440
ECFP	434	477
EGFP	489	508
EYFP	514	527
DsRed	558	583

Promoter

The concentration of GFP within a cell is a critical factor for its detection. Niswender, Blackman et al. (1995) estimated that intracellular concentrations must be at least 1 μ M for wild-type GFP to be detected by epifluorescence microscopy, laser confocal microscopy, or two-photon microscopy. These considerations indicate that fairly strong promoters must drive the expression of GFP, although mutant GFPs that fluoresce brighter may be detected at lower concentrations.

The promoter is a nucleotide sequence upstream of a gene that acts as a signal for RNA polymerase binding. It controls the first stage of gene expression (attachment of an RNA polymerase enzyme to the DNA) and determines the rate at which mRNA is synthesized. In other words, the promoter marks the point at which the transcription of gene should start (Brown 1995). Strong promoters are those that can sustain a high rate of transcription; they control genes whose translation products are required in large amounts by the cell.

Autofluorescence

Most cellular autofluorescence occurs in the excitation and emission wavelengths of GFP, thus background autofluorescence can obscure GFP signals (Aubin 1979; Niswender,

Blackman et al. 1995). This problem can be dealt with by selecting appropriate filters, or by using new variants of GFP that excite or emit at different wavelengths than that producing the problematic autofluorescence. Also, targeting of GFP fusion proteins to the nucleus, when possible, may help, since in general there is little autofluorescence in nuclei (Aubin 1979).

Posttranslational modification and GFP maturation

The posttranslational modification of GFP presumably involves initial folding of the apoprotein into an active conformation, to allow the cyclization and oxidation reactions that form the chromophore (Cody, Prasher et al. 1993; Cubitt, Heim et al. 1995; Heim, Prasher et al. 1994). The mature protein must then be correctly folded to maintain its fluorescent properties, to protect the chromophore from solvent effects (Ward, Cody et al. 1980).

2.2.3 Cavity Ring Down Spectroscopy

2.2.3.1 Background

Cavity Ring Down Spectroscopy (CRDS) is a way of making ultra-sensitive absorption measurements, particularly with gas-phase samples. The origin of the technique can be traced back to the early 1980s (Anderson, Frisch et al. 1984; Herbelin, McKay et al. 1980; Herbelin and McKay 1981; Kwok, Herbelin et al. 1982), and the applications have only recently been reviewed (Paul and SayKally 1997; Scherer, Paul et al. 1997). Since the technique has the ability to measure small fractional absorptions (down to sub-ppm levels per pass), it can benefit a broad area in the analytical-, physical-, and engineering communities.

The principle of CRDS is based on absorption spectroscopy. In a conventional absorption measurement, the intensity of light transmitted through a sample (I) is compared to the transmission without the sample (I_0) according to Beer-Lambert Law (Lajunen and Peramaki 2004):

$$I = I_0 \exp(-\sigma(\nu)LN). \quad (2-13)$$

where σ is the cross section of the absorbing species at the frequency ν of the measurement in $\text{molecule}^{-1}\cdot\text{cm}^2$ (sometimes also written as α , called ‘absorption coefficient’, unit is cm^{-1}), L is the path length of the absorbing medium in cm, and N is the number density of the absorbing species in $\text{molecule}\cdot\text{cm}^{-3}$ (or sometimes means concentration, dimensionless).

For gas phase analyte (Lajunen and Peramaki 2004),

$$\sigma(\nu) = S(\nu)/(\pi\gamma(\nu)) \quad (2-14)$$

where S is the line strength in $\text{cm}^{-1}/(\text{molecule}\cdot\text{cm}^{-2})$, γ is the half linewidth, which varies as gas pressure change, in $\text{cm}^{-1}/\text{atm}$ (Rothman, Rinsland et al. 1998).

The smallest number density that can be determined by this method is given by (Lajunen and Peramaki 2004):

$$N_{\min} = (\Delta I/I)_{\text{noise}} / (\sigma(\nu) \cdot L) \quad (2-15)$$

where $(\Delta I/I)_{\text{noise}}$ is the smallest fractional change in light intensity that can be detected.

Therefore, to maximize sensitivity, the analyte should have a large σ , long absorption path length L , and the small $(\Delta I/I)_{\text{noise}}$. For a particular molecule, the sensitivity and accuracy are thus limited by the system physical size and amplitude noise of light sources.

CRDS, on the other hand, is not affected by the laser noise $(\Delta I/I)_{noise}$ because it only measures "time". In a cavity ring down measurement, a laser pulse is coupled into an optical resonator, formed by two ultra-high reflectivity mirrors separated by a distance d . Light inside the cavity is reflected by the mirrors many times, the tiny amount of light leaking out of the cavity at each reflection is the ring down signal. Figure 2.10 shows a simple schematic of a typical experimental setup. Light from a laser is coupled into the cavity by mode-matching optics. A photo detector detects the transmitted light through the cavity. And then the signal is processed by a computer.

Figure 2.10 Schematic of a typical cavity ring down system.
M1, M2: High reflective mirrors; PD: Photodetector; PC: Computer.

This signal decays exponentially with time (Busch 1999),

$$I = I_0 \exp(-t/\tau) \quad (2-16)$$

where I_0 is the output light pulse intensity after one cycle through the cavity; t is random time; τ is cavity ring down time, when the output light equals to $1/e$ of I_0 . This decay arises from losses in the mirror coatings and absorption by the sample (normally is gas)

contained between the mirrors. The round trip loss A (in percent) and Γ (in dB) can be obtained as (Busch 1999),

$$A = \frac{I_0 - I_0 \exp(-t_r/\tau)}{I_0} = 1 - \exp(-t_r/\tau), \quad (2-17)$$

$$\Gamma = 10 \log I_0 - 10 \log(I_0 \exp(-t_r/\tau)) = 4.34 t_r/\tau, \quad (2-18)$$

where t_r is the light pulse round trip time in the cavity.

When light is tuned away from the molecular resonance or the cavity is empty, the ring down time, τ_{empty} , is determined by (Busch 1999)

$$\tau_{empty} = \frac{d}{c(1-R)} \quad (2-19)$$

where d is the physical separation of the two cavity mirrors; c is the speed of light, R is the reflectivity of the mirrors (assumed to be the same for both mirrors). When there is sample present inside the cavity, the ring down time, $\tau(\nu)$, is then determined also by absorption by the sample (Busch 1999),

$$\tau(\nu) = \frac{d}{c(1-R + \sigma(\nu) \cdot N \cdot d)} \quad (2-20)$$

where $\tau(\nu)$ is the ring down time at frequency ν ; $\sigma(\nu)$ is the absorption cross section of the particular molecules that absorb light at frequency ν ; N is their number density, that can be calculated from the following equation (Busch 1999):

$$c\sigma(\nu)N = \frac{1}{\tau(\nu)} - \frac{1}{\tau_{empty}} \quad (2-21)$$

Romanini and Lehmann (1993) have shown that the ring down cavity (RDC) can be viewed as having an effective path length $L = d/(1-R)$. Since commercially available

mirrors can have reflectivity of 99.999%, the effective optical path length of the cavity is increased by 10^5 times over the physical dimensions of the device. Thus an absorption path length of 1,000–100,000 meters is achieved in the cavity with a 1-m physical length. This means that CRDS can effectively provide an internal gain of more than 10^3 for absorption measurement.

2.2.3.2 Fiber Cavity Ring Down Spectroscopy

Driven by its high sensitivity, CRDS has made a rapid progress within the past decade (Busch 1999), with more than 300 publications worldwide (Atkinson 2003; Berden, Peeters et al. 2000), though little improvement has been made in applications beyond localized detection. On the other hand, the versatility of optical fiber has broadened its usage into a variety of new sensing fields (Wolfbeis 2002), but its use is limited in the common single pass measurement techniques. These two technologies have recently been combined as fiber cavity ring down spectroscopy (FCRDS), which uses an optical fiber loop or resonator as the ring down cavity (Brown, Kozin et al. 2002; Gupta, Jiao et al. 2002; Stewart, Atherton et al. 2001; Tarsa, Brzozowski et al. 2004; von Lerber and Sigrist 2002a).

It should be noted that the fiber loop configuration has several advantages over the conventional mirror cavity ring down setup.

Single mode fiber (SMF), compatible with a variety of inexpensive, rugged components in telecom systems, has low attenuation and supports only the lowest order transverse mode, making it appropriate for use in CRDS resonators, where minimal loss is required

and single mode excitation improves stability. Therefore, mode matching optics is not required in the fiber loop system in order to excite only longitudinal modes (Romanini, Kachanov et al. 1997; Romanini 1998), as mode matching is not a trivial task.

For light to travel inside the cavity, formed by two ultra-high reflectivity mirrors separated by a distance d , with minute loss every round trip, the cavity has to be aligned so that the cavity resonant frequency is overlapped with light frequency. Cavity resonant frequencies and cavity mode spacing are given by (Svelto and Hanna 1989),

$$\nu = m \frac{c}{2nd} \quad (2-22)$$

$$\Delta\nu = c/(2nd) \quad (2-23)$$

where m is a positive integer, c is the speed of light in vacuum, and n is refractive index of media inside the cavity. To couple laser light into cavity resonance, the cavity length has to be integral number of half-wavelength of the light. For short cavity, its cavity mode spacing is too wide, the cavity length has to be carefully aligned so that the cavity resonant frequency is overlapped with light frequency, e.g. 1-m fiber cavity, n is ~ 1.5 , $\Delta\nu = 100$ MHz. Compared with the laser light linewidth, it may need tedious cavity alignment and complex components to couple laser light into the cavity resonances (Figure 2.11 shows the relationship of cavity resonance curves and laser light spectrum). While for long fiber loop system, >10 -m loop can be used, the cavity mode spacing is < 10 MHz, hence light coupling is not an issue. For trace gas monitoring at atmospheric pressure, typical (pressure broadened) absorption linewidth is of the order of several GHz, several hundreds loop cavity modes will overlap with the gas linewidth. Furthermore, conventional cavity is < 1 m long with a round trip time of about 10 ns, so the input laser pulse width has to be less than 10 ns to avoid overlapping of adjacent signal. In contrast,

the fiber loop can be 10–100 m long, relaxing the constraints on the pulse width and avoiding the use of high-speed detectors. Also, the long distance transmission capacity of SMF combined with its flexibility can be exploited to expand the utility of CRDS to a variety of novel conditions, e.g. multipoint distributed sensing, and remote sensing.

Figure 2.11 Relationship of cavity resonance curves (dash line) and laser light spectrum (solid line). (a) Cavity mode spacing is wider than laser linewidth, (b) Cavity mode spacing is narrower than laser linewidth.

The evolution of the fiber cavity ring down spectroscopy (FCRDS) has led to a greatly diversified technique (classified by configurations of ring down cavities) including fiber end-coated CRDS (von Lerber and Sigrist 2002a, b), fiber Bragg grating (FBG) CRDS (Gupta, Jiao et al. 2002), and fiber coupler CRDS (Tarsa, Brzozowski et al. 2004).

Initially, the fiber ring down cavity was formed by two high-reflectivity mirrors placed adjacent to the polished fiber ends, and index-matching fluid was used to help reduce coupling losses (Stone and Marcuse 1986). This technique is very sensitive to mirror alignment and quality (Marcuse and Stone 1986).

Within the past few years, cavities have been produced by deposit of a highly reflective dielectric coating on the polished fiber ends (von Lerber and Sigrist 2002a). Again, this technique is also sensitive to the coating quality, and light coupling between fiber and coating.

In 2002, a fiber cavity employing FBGs was introduced (Gupta, Jiao et al. 2002). Since the FBG is within the fiber core, it is not affected by fiber polishing and is always normal to the fiber axis, so cavity alignment is not a problem. And because of their widespread utility in the telecom industry, FBGs are commercially available and cost effective. However, the operation wavelength range of such a cavity is limited because it must fall within the grating bandwidth and it critically depends on closely matched gratings.

In 2002, Brown, Kozin et al. (2002) reported their progress on a fiber loop ring down technique developed for liquid phase detection. Two ends of the fiber were connected to form a loop using splice connector, within the gap of the connector was the liquid analyte. Light was coupled in/out of the loop from bending regions of the loop. The configuration is simple, but the light intensity coupled in/out of the loop is very weak (10^{-8} for coupling in, 10^{-4} for coupling out), so 3000–6000 laser shots have to be averaged to remove the noise and get the ring down waveform.

Also, Tarsa et al. (Tarsa, Rabinowitz et al. 2002; Tarsa, Brzozowski et al. 2004; Tarsa, Rabinowitz et al. 2004) reported their study on FCRDS to measure strain and 1-octyne. Two couplers were used in place of the mirrors used in conventional CRDS to couple light in/out of the cavity. A tapered fiber was spliced inside the cavity as sensing region.

All the above are passive cavity ring down techniques. These techniques normally can only be used in simple evanescent field interaction in a medium of similar index to that of the fiber cladding and core (the loss of the cavity is small). To achieve long ring down time, a very high finesse cavity is required and the intrinsic loss of the cavity should be very low. With an active fiber loop cavity including gain, these requirements are not necessary any more. Stewart et al. (Stewart, Atherton et al. 2001, 2004; Stewart, Shields et al. 2004) reported their work of active cavity ring down technique, in which an erbium doped fiber amplifier (EDFA) was used in the fiber cavity configuration for gas phase absorption measurement. The EDFA can compensate for the intrinsic loss of cavity. The problem is then transferred from the intrinsic loss of the fiber loop to stable pumping and gain of the EDFA, and thus the variation of the ring down time.

Chapter 3 Nutrient Stress Analyzer–Development

3.1 Introduction

Sub-optimal management of crop nutrient poses a major yield constraint in commercial crop production for food, medicine or valuable plant extracts. Optimal mineral nutrient management is also of key importance for the production of quality food to meet human nutritional requirements (Welch 2002). Conventional approaches to ascertaining plant nutrient status rely primarily on determining total elemental content in plant tissues and quick field tests on plant sap and fluids (Jones 1998). These methods are time consuming, tedious, expensive and involve destructive sampling of plant parts. A further disadvantage of conventional methods is that the interpretations of total elemental content in tissues do not generally include consideration of the distribution and form of the element within cells. For example, iron (Fe) can exist in trivalent or divalent forms in plant tissues or complex within iron-containing proteins and enzymes, thus influencing its bioavailability for metabolic reactions and chlorophyll biosynthesis. In lettuces exhibiting distinct Fe-deficient symptoms, 93–604 $\mu\text{g g}^{-1}$ Fe was found in the tissues compared with 130–1468 $\mu\text{g g}^{-1}$ Fe in healthy plants (Roorda van Eysinga and Smilde 1971). This phenomena has also been observed in *Spathiphyllum* where Fe-deficient leaves manifesting interveinal chlorosis contained iron at levels similar to that of Fe-sufficient leaves exhibiting no morphological symptoms (Lang, Rosenfield et al. 1990; Yeh, Lin et al. 2000). Hence, simple analysis of total elemental content may be of limited value for diagnosis of nutrient adequacy within plant tissues.

The increasing emphasis on non-destructive testing methods has prompted the development of devices such as chlorophyll meter, which directly measures leaf transmittance and relates it with nitrogen (N) status in the plant (Piekielek and Fox 1992; Schepers, Francis et al. 1992). Research has also focused on finding specific wavelengths or vegetation indices, from the leaf reflectance spectrum, which can characterize chlorophyll or pigment status (Carter 1993; Daughtry, Walthall et al. 2000; Richardson, Duigan et al. 2002; Sims and Gamon 2002; Smith, Steven et al. 2004). In previous studies (Carter 1993; Richardson, Duigan et al. 2002), plants were subjected to prolonged periods of stress and spectral data was acquired at the point where physical stress symptoms became apparent. In other work (Chiera, Thomas et al. 2002), seedlings were subjected to stress shortly after germination, a stage where the plants are most sensitive and would display visual symptoms very rapidly. Such approaches preclude pre-visual determination of plant stress. Therefore, to predict and distinguish different stresses non-destructively in field, a nutrient stress analyzer based on reflectance spectroscopy was proposed.

3.2 Hardware

Figure 3.1 shows the nutrient stress analyzer. For field application, a portable system is shown. It is similar to the laboratory setup, except the laptop is replaced by a personal digital assistant (PDA).

The reflectance spectra (range from 380–800 nm) were acquired through an optical fiber cable (R-series Fiber Optic Reflection Probes, OceanOptics, USA). One end of this bifurcated cable was connected to a spectrometer (S2000-UV-VIS, OceanOptics, USA) with a spectral resolution of 1.5 nm. The other end was connected to a tungsten halogen

lamp (LS-1, OceanOptics, USA), which served as the light source. Figure 3.2 shows the spectral irradiance distribution of the tungsten halogen lamp. The common end of the cable served as the probe head comprising seven fibers with one fiber in the center and the rest arranged along the circumference (Figure 3.3). Light from a tungsten halogen lamp illuminated the leaf via the center fiber of the probe, and the reflected light from the adaxial (upper) leaf surface was collected by the six surrounding fibers and directed to the spectrometer. The probe head was improved to avoid interference from ambient light influence and to ensure that reflectance spectra were collected at the same distance (5 mm) above the leaf surface and over the same leaf surface area (disc diameter 6 mm). The disc diameter was determined by the probe diameter. The distance—5 mm, was chosen to allow suitable light spot size shined on the leaf surface, while not too far away from the surface to collect very dim light. As the fiber's numerical aperture (NA) is 0.22 (OceanOptics, USA), the light spot size shined on the leaf surface will be around 800 μm . While typical plant cell diameter is $\sim 100 \mu\text{m}$ (Hopkins 1999), this will allow a reasonable surface area to be diagnosed. A button was added to the probe for data capturing control. The spectrometer data was transferred to a laptop via an A/D card (DAQCard-700, National Instruments, USA) for further processing and analysis.

Figure 3.1 Nutrient stress analyzer. (a) Laboratory setup, (b) Portable system.

Figure 3.2 Spectral irradiance distribution of the tungsten halogen lamp.
(<http://www.oceanoptics.com/products/lsl.asp>)

Figure 3.3 Probe structure.
A-A: the probe cross section.

3.3 Software

Figure 3.4 shows the flow chart of the data acquisition & process program developed for the system. First, a reflective spectrum from a reference white surface (WS-1 Diffuse Reflection Standard, OceanOptics, USA) was collected. Next, a reflective spectrum of the tested leaf was collected. The leaf reflectance spectrum was then calculated as:

$$R = \text{Leaf reflective spectrum} / \text{Reference reflective spectrum.} \quad (3-1)$$

The leaf reflectance spectrum was analyzed to set criteria for different nutrient stress. Finally, according to the criteria, plant nutrient status was indicated.

Figure 3.4 Flow chart of data acquisition & process program.

There are many ways to analyze a reflectance spectrum, using either specific wavelengths or the whole spectrum. But in the case of a spectrum (range from 380–800 nm), how do we decide which specific wavelengths to look at? On the other hand, spectrum matching can be tedious and time consuming. Hence, three methods were thought of: first, obvious spectrum features were checked—red edge; second, statistic method (partial least squares regression) was used to look for specific wavelengths that can present information for stress detection; third, checking on the whole spectrum was simplified by using CIELAB color system—the spectrum was transformed into three color values.

3.3.1 Obvious Spectrum Feature—Red Edge Inflection Point (REIP)

To look at obvious spectrum features, red edge was studied. The phenomena of red edge is the result of a combination of strong absorption by chlorophyll in the red region and strong reflectance in the near infrared region (800–1100 nm) due to multiple scattering in the leaf mesophyll and absence of absorption by pigments (Milton, Eiswerth et al. 1991; Woolley 1971). Red edge is the maximum slope in reflectance spectrum, normally found in the range from 680 nm to 750 nm. As shown in Figure 3.5, there is wavelength shift of the red edge as the spectra change.

Figure 3.5 Reflectance spectra from different-color leaves showing red edge shift.

To clearly monitor the shift, red edge inflection point (REIP) was introduced. REIP is the maximum first derivative of the reflectance spectrum in the red edge region. Figure 3.6 shows a typical reflectance spectrum and its first derivative spectrum. The wavelength

position and magnitude of REIP have been used in previous studies to determine the levels of radiation absorbed during photosynthesis (Hall, Huemmrich et al. 1990) and leaf pigment content (Curran, Dungan et al. 1991; Lamb, Steyn-Ross et al. 2002; Pinar and Curran 1996); to discriminate crop type and maturity (Collins 1978) and to estimate the severity of stress (Demetriades-Shah, Steven et al. 1990; Ferns, Zara et al. 1984) and crop condition (Boochs, Kupfer et al. 1990; Hoque and Hutzler 1992).

Figure 3.6 Reflectance spectrum (dark) and its first derivative spectrum (grey). The dash line shows the red edge inflection point in the two spectra. The first derivative spectrum has been normalized for viewing.

3.3.2 Specific Wavelengths

Wavelengths other than the REIP wavelength were also checked to find ‘important’ wavelengths which can help to set stress criteria. Prior to checking, the raw reflectance spectrum needs to be pre-processed.

3.3.2.1 Reflectance Spectrum Pre-processing

Reflectance from the leaf composed of two components, $R = R^E + R^I$ (McClendon and Fukshansky 1990a, b). The external reflectance, R^E , is the direct reflection at the air-epidermis interface because of the mismatch of the refractive index and it is constant in the 380–780 nm range. The inner reflectance, R^I , carries pigment information, and depends on the scattering and absorbing properties of the leaf inner layers. Within the absorption bands and at high chlorophyll content, R^I vanishes since the penetrating light is immediately absorbed. In these bands, the leaf reflectance is only due to R^E . Therefore, spectral regions exist where the measured reflectance equals to R^E , and the R^I can be obtained by subtracting R^E from the total reflectance R (Maccioni, Agati et al. 2001). Because the maximum pigment absorption occurs in blue region, $R^E = R_b$, where R_b is the average reflectance of 380–480 nm. R_b has a value of about 0.05 and shows no correlation with chlorophyll (Gitelson and Merzlyak 1996; Lichtenthaler, Gitelson et al. 1996). For $380 \leq \lambda \leq 780$ nm,

$$R^I(\lambda) = R(\lambda) - R_b \quad (3-2)$$

In regions outside the chlorophyll absorption band, e.g. at 750 nm and above, R^I is caused by scattering effect, while inside chlorophyll absorption bands, part of light is absorbed, thus, by normalizing $R^I(\lambda)$ with $R^I(\lambda_0)$, the inner leaf scattering effect was removed. The normalized inner reflectance, NR^I , removes the effects of external reflectance and inner leaf scattering, and is linearly correlated with the logarithm of chlorophyll content (Maccioni, Agati et al. 2001). For $380 \leq \lambda \leq 780$ nm, $\lambda_0 \geq 750$ nm,

$$NR^I(\lambda, \lambda_0) = R^I(\lambda) / R^I(\lambda_0) \quad (3-3)$$

Figure 3.7 shows the reflectance spectra, inner spectra and normalized inner spectra of two leaves with the same chlorophyll content. It can be seen that even with the same chlorophyll content, the two reflectance spectra were quite different from each other (Figure 3.7a), while after transformation into normalized inner reflectance, nearly no difference was observed (Figure 3.7c). Therefore, it is more accurate to analyze the transformed normalized inner reflectance spectrum. Note that, the normalized inner reflectance spectrum was not used in REIP method, because the calculated REIP wavelength positions from reflectance spectrum and from normalized inner reflectance spectrum are the same.

Figure 3.7 Reflectance (a), inner reflectance (b) and normalized inner reflectance (c) spectra from two leaves with the same chlorophyll content.

3.3.2.2 Partial Least Squares Procedure

Partial least squares (PLS) regression is a powerful tool in chemometrics and other fields. It is developed for modeling M observations with J x -variables denoted by x_j ($j=1, 2, \dots, J$), and K y -variables y_k ($k=1, 2, \dots, K$). These data form the two matrices $\mathbf{X}$ and $\mathbf{Y}$ of dimensions $(M \times J)$ and $(M \times K)$, respectively. In PLS regression, x -variables that are important for the modeling of $\mathbf{Y}$ are identified by large regression coefficients ($\mathbf{B}$ -matrix). However, an x -variable may also be important for the modeling of $\mathbf{X}$. A summary of the importance of an x -variable for both $\mathbf{X}$ and $\mathbf{Y}$ is given by variable influence on projection (VIP). The following equations show the PLS modeling algorithm (Wold, Sjöström et al. 2001). The PLSR model finds a few new variables called x -scores and denoted by $\mathbf{T}$. They are estimated as linear combinations of the original x -variables with the coefficients “weights”, $\mathbf{W}^*$ (3-4). The x -scores model $\mathbf{X}$ (3-5) and also are predictors $\mathbf{Y}$ (3-6). The y -scores, denoted by $\mathbf{U}$, model $\mathbf{Y}$ (3-7). Equation (3-6) can be rewritten into (3-8), which represent a regression model. The PLS regression coefficients are denoted by $\mathbf{B}$ (3-9). VIPs can be calculated by weighted sum of squares of x -weights ($\mathbf{W}^*$).

$$\mathbf{T} = \mathbf{X}\mathbf{W}^* \quad (3-4)$$

$$\mathbf{X} = \mathbf{T}\mathbf{P}' + \mathbf{E} \quad (3-5)$$

$$\mathbf{Y} = \mathbf{T}\mathbf{C}' + \mathbf{F} \quad (3-6)$$

$$\mathbf{Y} = \mathbf{U}\mathbf{C}' + \mathbf{G} \quad (3-7)$$

$$\mathbf{Y} = \mathbf{X}\mathbf{W}^*\mathbf{C}' + \mathbf{F} = \mathbf{X}\mathbf{B} + \mathbf{F} \quad (3-8)$$

$$\mathbf{B} = \mathbf{W}^*\mathbf{C}' \quad (3-9)$$

where $\mathbf{P}$ is x -loading-matrix, $\mathbf{E}$ is x -residual-matrix, $\mathbf{C}$ is y -weight-matrix, and both $\mathbf{F}$ and $\mathbf{G}$ are y -residual-matrices.

The PLS procedure (SAS 8.2, SAS Institute, USA) (SAS Institute Inc. 2000) was used to calculate B-matrix and VIP values to find the important wavelengths whose NR^I were highly correlated with Log(Chl). If no specific ‘important’ wavelengths were found, the whole spectrum was checked.

3.3.3 Whole Spectrum Simplification—CIELAB Color Values

In the case of checking on the whole spectrum, instead of looking at hundreds of wavelengths, color system was used, which can represent the whole spectrum information in three values. Using equations shown in section 2.2.1.2, $L^* = 116 \times (Y/Y_n)^{1/3} - 16$ (2-5), or $L^* = 903.3 \times Y/Y_n$ (2-6), $a^* = 500 \times (f(X/X_n) - f(Y/Y_n))$ (2-7), and $b^* = 500 \times (f(Y/Y_n) - f(Z/Z_n))$ (2-8), the normalized inner reflectance spectrum was converted into three color values: L^* , a^* , and b^* . The three values provided a three dimensional perception of the leaf color, which is highly correlated to chlorophyll content, and nutrient content. Figure 3.8 shows a normalized inner reflectance spectrum and its corresponding L^* , a^* , and b^* values.

Figure 3.8 A normalized inner reflectance spectrum and its corresponding L^* , a^* , and b^* values.

3.4 Conclusion

As the most straightforward non-destructive method, reflectance spectroscopy was used to establish pre-visual spectral cues for nutrient stressed plants. The expected nutrient stress analyzer should be:

- Able to detect different nutrient stress early enough for remedial actions to be taken
- Non-destructive
- Remote accessible
- Portable
- User friendly

Based on these, both hardware and software parts were proposed for reliable data acquisition and analysis. While different nutrient stresses provide different information on the spectrum, some might be easily seen from obvious spectrum features, some might relate to specific wavelengths, others might affect the overall spectrum. The aim is to find these effects and correlate them to different nutrient stresses for stress prediction. In chapter four, three different types of nutrients are studied for setting stress criteria.

Chapter 4 Nutrient Stress Analyzer–Application

Reflectance spectroscopy and strategies for spectral analysis over the visible range from 380–780 nm were used to provide diagnostic information on the calcium, iron and phosphorus status of *Brassica chinensis* L. var *parachinensis* (Bailey) grown under hydroponics conditions. The physiological and morphological changes were also tracked in relation to spectral changes to evaluate the diagnostic value of optical signatures in assessing the nutritional status of plants.

4.1 Introduction

Different nutrient elements have different characteristic stress symptoms within the plant (Hale, Orcutt et al. 1987). To develop criteria for nutrient stress detection and test the home-developed nutrient stress analyzer, three types of nutrients (calcium, iron and phosphorus) were studied.

Calcium (Ca) is an important nutrient for plant growth and functions primarily as a key component in cell wall. It is also required for the physical integrity and permeability of the cell membrane. Ca is able to form coordinate bonds and establish stable but reversible intra- and inter-molecular linkages especially in the cell wall and at the surface of the membranes (Hewitt 1963; Rains 1976). A deficiency in Ca usually results in smaller plants with spotted or necrotic (dead) areas on leaves. Figure 4.1 shows the Ca deficiency (–Ca) symptom on soybean. Since Ca is an immobile nutrient element and cannot be

translocated within the plant, deficiency symptoms will appear in the growing terminals first (Hale, Orcutt et al. 1987).

Figure 4.1 Control and Ca-deficient soybean leaves.
(http://www.usu.edu/cpl/research_hydroponics4.htm)

Iron (Fe) mineral nutrition presents difficulties in crop management due to the low solubility of both ferrous (Fe^{2+}) and ferric (Fe^{3+}) ion compounds, particularly in calcareous soils (Chen and Barak 1982). Amendment of soil pH may also cause available Fe to precipitate due to overliming, thus leading to Fe deficiency or lime-induced chlorosis. Plant response to Fe deficiency ($-\text{Fe}$) is manifested in a plethora of morphological and biochemical changes (Kosegarten, Wilson et al. 1998; Mariotti, Ercoli et al. 1996; Yeh, Lin et al. 2000; Zaharieva, Gogorcena et al. 2004). Typical consequences of Fe deficient states include interveinal chlorosis of apical leaves and the structural disorganization of chloroplasts and mitochondria (Briat, Fobis-Loisy et al. 1995; Thoirion, Pascal et al. 1999). Besides yellowing, plant growth can also be hampered (Kosegarten, Wilson et al. 1998). In severe cases, Fe deficiency can result in extensive chlorosis,

necrosis, arrested growth or even death. Figure 4.2 shows the Fe deficiency symptom on soybean.

Figure 4.2 Fe-deficient soybean leaves.
(http://www2.ctahr.hawaii.edu/tpss/research_extension/rxsoil/fe.htm)

Phosphorus (P) is a key plant macronutrient absorbed by plants mostly in the monovalent orthophosphate form H_2PO_4^- . Under cytoplasmic pH conditions, inorganic phosphate (P_i) remain largely as the primary or secondary orthophosphate anions, H_2PO_4^- and $\text{H}_2\text{PO}_4^{2-}$. Intracellular phosphorus can be complexed into organic forms as a structural component of key macromolecules including nucleic acids, phospholipids, certain amino acids and coenzymes. Attachment of P_i molecules to one another to form high energy pyrophosphate bonds in ATP and to different sugars contributes to the significant role of phosphorus in energy transfer and in providing the necessary metabolic energy in photosynthesis and respiration (Assuero, Mollier et al. 2004; Chiera, Thomas et al. 2002; Mikulska, Bomsel et al. 1998; Milton, Eiswerth et al. 1991). The symptoms of P deficiency can be explained on the basis of its multifacrious functional roles in plants.

Since P is an essential component of genetic material, cell division and expansion are adversely affected by P deprivation (Assuero, Mollier et al. 2004). P-deficient plants typically display weak or stunted growth. Lack of P also hinders metabolic processes leading to sugar buildup, which is associated with anthocyanin formation and the development of purplish tinge in leaves and stems. P is not a component of chlorophyll but its deficiency may lead to high chlorophyll content when nitrogen is present in abundant supply; hence some P-deficient leaves display a dark green color (Chiera, Thomas et al. 2002; Juszczuk, Malusà et al. 2001). Figure 4.3 shows the P deficiency (–P) symptom on soybean. Being a mobile element, P can be reabsorbed from mature and senescing organs to young developing parts, hastening the senescence of older leaves (Usuda 1995). Visual symptoms of P deficiency may be difficult to distinguish in some plants where some studies showed that *Spathiphyllum* grown in P-deficient media for 110 days showed no visual symptoms although there was a reduction in chlorophyll content (Yeh, Lin et al. 2000).

Figure 4.3 Control and P-deficient soybean leaves.
(http://www.usu.edu/cpl/research_hydroponics4.htm)

4.2 Materials and Methods

4.2.1 Plants Sample Preparation

The leafy green vegetable, *Brassica chinensis* L. var *parachinensis* (Bailey), was used in our experiments. Figure 4.4 shows the plant sample preparation procedure.

In the first experiment, seeds were sown in sponge cubes and germinated in water in the dark at 25°C for three days. The seedlings were then moved to the greenhouse for gradual hardening to outdoor light and temperature conditions. One week after germination, the seedlings were transferred to a re-circulating hydroponic system (Figure 4.5) and grown in complete nutrient solution for twenty days until they reached the ‘6-leaf stage’. Two hundred plants were then divided into two equal groups and transferred to two types of nutrient media respectively for 7 days (Figure 4.6). In the control (Ctrl) group, plants continued to be maintained in complete nutrient solution where they were supplied with all the essential nutrient elements. In the Ca-deficient (–Ca) group, plants were subjected to calcium stress by growing them in nutrient solution lacking calcium.

The sample preparation procedure was similar in the second experiment till plants reached the ‘6-leaf stage’, 144 plants were divided equally into Ctrl and Fe deficient (–Fe) groups; in the third experiment, 192 plants were divided equally into Ctrl and P deficient (–P) groups. In the Ctrl group, the plants continued to be maintained in complete nutrient solution where they were supplied with all the essential nutrient elements. In the –Fe group, plants were subjected to Fe stress by growing them in exactly the same nutrient solution as for Ctrl group except that the Fe component was excluded. In the –P group,

the nutrient solution was formulated to eliminate $\text{PO}_4\text{-P}$ without changing the concentrations of the other essential elements.

In all three experiments, the electrical conductivity (EC) and pH levels of all nutrient solutions were maintained at 1600–1800 $\mu\text{S}/\text{cm}$ and pH 6.0–6.4 respectively as measured at 25°C throughout the experimental period.

Figure 4.4 Plant sample preparation procedure.

Figure 4.5 Plants in re-circulating hydroponic system.
Arrows indicate nutrient solution flow direction.

Figure 4.6 Plant samples in different types of nutrient media.

4.2.2 Reflectance Measurement

Reflectance spectra were collected daily from the youngest fully expanded leaf of each plant at around 1800 hours using nutrient stress analyzer shown in Figure 3.1a. Collection positions were located at the lower base of the leaves (Figure 4.7, see appendix for detail), because a tissue element is displaced away from the leaf base as a result of production of younger tissue below it (Bernstein, Silk et al. 1993). Therefore, deficient symptoms were expected to first appear around the basal region of the leaf. Initial spectral characteristics of nutrient sufficient plants maintained in complete solution were captured on Day 0 of the experiment. Subsequently, these plants were transferred to the various treatment solutions and spectral data was collected every 24 hours for 7 days.

Figure 4.7 Spectra collection position.

4.2.3 Chlorophyll Extraction

The methods used for chlorophyll extraction were described in Porra, Thompson et al. 1989. For chlorophyll determinations, circular leaf discs were punched from the same area where reflectance spectra were acquired. Each disc was immediately put in a 1.5-ml Eppendorf tube containing 1 ml of absolute methanol and pigments were extracted in the dark for 24 hours at room temperature. Following centrifugation at 13,000 rpm for 1 min, the clear supernatant was transferred to a polystyrene cuvette for spectrophotometric measurement of absorbance at 652, 665, and 750 nm. The concentrations ($\mu\text{g}/\text{cm}^2$) of chlorophylls *a*, *b* and *a + b* were calculated from the following equations (Porra, Thompson et al. 1989):

$$\text{Chla} = 16.29A^{665} - 8.54A^{652} \quad (4-1)$$

$$\text{Chlb} = 30.66A^{652} - 13.58A^{665} \quad (4-2)$$

$$\text{Chl}(a + b) = 22.12A^{652} + 2.71A^{665} \quad (4-3)$$

where A^{665} and A^{652} were the measured absorbance at 665 nm and 652 nm minus the absorbance at 750 nm, respectively.

4.2.4 Nutrient Content Measurements

4.2.4.1 Ca Content Measurements

In the first experiment, four determinations of total Ca content for each treatment group were made daily. For each measurement, 2 g fresh weight leaf tissue was obtained from the terminal regions of three randomly selected plants and combined to form one composite sample. The composite sample was dried in a forced-air oven at 80°C for 24 hours. Total Ca content for all composite samples were then determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) according to Isaac and Johnson (1998).

4.2.4.2 Fe Content Measurements

In the second experiment, three determinations of total Fe content for each treatment group were made daily. The same composite sample preparation and measurement methods, used for Ca content measurement, were used for Fe content measurement.

4.2.4.3 P Content Measurements

In the third experiment, four determinations of acid-extractable P content for each treatment group were made daily. For each measurement, 2 g fresh weight leaf tissue was obtained from the terminal regions of three randomly selected plants and combined to form one composite sample. The composite sample was dried in a forced-air oven at 80°C for 24 hours, and then was ground to a fine powder using a mortar and pestle. One hundred mg of the dried sample was extracted in 25 ml of 2% acetic acid for 30 min (Miller 1998), then filtered through 11 µm medium retention Whatman filter paper. PO₄-P in the extract was determined by the vanadomolybdophosphoric acid colorimetric method

with absorbance measured at 470 nm (Eaton, Clesceri et al. 1996). Correlation with a standard curve derived from analar grade anhydrous KH_2PO_4 permits calculation of the $\text{PO}_4\text{-P}$ content in the extract and per unit dry weight leaf tissue.

4.3 Results and Discussion

4.3.1 Ca Stress Detection

4.3.1.1 Morphological and Physiological Effects of Ca Stress

No visual difference was observed between Ctrl and –Ca plants throughout the experiment. The plant physiological responses to Ca stress were monitored in terms of elemental and chlorophyll content. According to Demarty, Morvan et al. (1984), Ca content in healthy vegetable plants are typically in the range of 10,000–30,000 ppm. As shown in Figure 4.8, the Ca content in Ctrl plants remained relatively constant around 20,000 ppm, while that in –Ca plants decreased to about 5,000 ppm by Day 5 and remained relatively unchanged at this level till the end of the experiment. **It was observed that the Ca content of –Ca plants fell below the typical sufficiency level of 10,000 ppm on Day 4 (which means that the Ca content difference between Ctrl and –Ca plants $\Delta[\text{Ca}] > 10,000$ ppm on day 4), and it might be interpreted that –Ca plants had likely entered into a deficiency state.** As shown in Figure 4.9, chlorophyll (Chl) content increased steadily with time but no significant difference was observed between Ctrl and –Ca plants. This implied that the lack of Ca did not affect leaf chlorophyll content, at least not within our experimental period.

Figure 4.8 Total Ca content in Ctrl and -Ca plants as a function of time. Mean values are denoted by closed or open circles and $\pm$ SE are indicated by the error bars.

Figure 4.9 Chlorophyll content in Ctrl and -Ca plants as a function of time. Mean values are denoted by closed or open circles and $\pm$ SE are indicated by the error bars.

4.3.1.2 Reflectance Spectral Features

In view of the functional roles of Ca in plants, it is anticipated that lack of Ca will result in structural changes to the plant cell wall. The loss of membrane integrity, particularly with respect to the thylakoid membranes into which chlorophyll pigments are embedded, would also impact on chlorophyll status and photosynthetic processes. Therefore, it would be best to identify optical feature(s) from the leaf reflectance spectrum that can be corroborated with early changes in leaf anatomical structure and chlorophyll status as a pre-visual means of detecting Ca deficiency. As stated in section 3.4.1, the phenomena of red edge is the result of a combination of strong absorption by chlorophyll in the red region and strong reflectance in the near infrared region (800–1100 nm) due to multiple scattering in the leaf mesophyll and absence of absorption by pigments (Milton, Eiswerth et al. 1991; Woolley 1971). Therefore, REIP method was used to find the stress criteria for Ca deficiency.

As shown in Figure 4.10, the REIP position was found to shift towards longer wavelengths in the Ctrl group as the plants matured and reached a relatively steady position around 714–715 nm from Day 4 onwards. On the other hand, after a slight initial shift towards longer wavelengths, the REIP of –Ca plants remained relatively unchanged around 708 nm from Day 2 onwards. An increase in chlorophyll content was expected to cause both deepening and broadening of the chlorophyll absorption feature and hence movement of red edge to longer wavelengths (Ferns, Zara et al. 1984; Pinar and Curran 1996). While this was true for plants grown in complete nutrient solution, this trend was not observed for –Ca plants. One possible reason is that chlorophyll content may increase as –Ca plants matured, the consequent red-shifting of REIP to longer wavelengths could be negated by concomitant blue-shifting of the REIP to shorter wavelengths that arise

from enhancement of light scattering in the near infrared region due to anatomical changes in the leaf. The opposing movements of the REIP attributed to chlorophyll increase and anatomical changes could be of similar magnitude, thus explaining the relatively constant position of the REIP from Day 2 till the end of the experimental period.

Figure 4.10 Red edge inflection points in Ctrl and –Ca plants as a function of time. Mean values are denoted by closed or open circles and $\pm$ SE are indicated by the error bars.

The difference in REIP values and Ca content between Ctrl and –Ca plants, $\Delta[\text{REIP}]$ and $\Delta[\text{Ca}]$ respectively, were computed and their relationship is shown in Figure 4.11. From the linear graph, it can be observed that when $\Delta[\text{Ca}] > 10,000\text{ppm}$, $\Delta[\text{REIP}] > 4\text{ nm}$. As indicated in section 4.3.1.1, from day4 (the fourth day since the plants were subjected to Ca-deficient nutrient solution) onwards, $\Delta[\text{Ca}] > 10,000\text{ ppm}$, plants entered Ca-deficient state. Thus it would be indicative that when $\Delta[\text{REIP}] > 4\text{ nm}$, the –Ca plants entered Ca-deficient state. **Alternatively, our results may be interpreted to mean that if the REIP**

position of a 24-day old plant (counting from 1 week of germination) does not reach or exceed 708 nm, the plant is in Ca-deficient state. Significantly, no visual deficiency symptoms were observed in –Ca plants throughout the experimental period. Hence, the REIP position of leaf reflectance represents a potentially attractive optical property for early, non-destructive diagnosis of Ca deficiency in *Brassica* plants.

Figure 4.11 Relationship between $\Delta[\text{REIP}]$ and $\Delta[\text{Ca}]$.

4.3.2 Fe Stress Detection

4.3.2.1 Morphological and Physiological Effects of Fe Stress

4.3.2.1.1 Morphological changes

Slight yellowing in the basal region of the youngest fully expanded leaf in a few –Fe plants was observed by day 2 but there were no differences between the veinal and interveinal regions. The characteristic leaf interveinal chlorosis became visually evident only from day 4 onwards. By the end of the experimental period, most –Fe plants had developed marked interveinal chlorosis in the young leaves but there were no indications of stunting of growth or any leaf necrosis. There was a degree of variation in plant response to Fe with some plants displaying chlorosis earlier and more extensively than others.

4.3.2.1.2 Physiological changes

An initial increase in Fe and Chl content in Ctrl plants grown under complete nutrient solution was observed in the first two days and then remained relatively unchanged till the end of the experimental period (Figure 4.12, 4.13). The Fe and Chl levels in Ctrl plants ranged from 100–137 ppm and 20–35 $\mu\text{g}/\text{cm}^2$ respectively. Compared with Ctrl plants, both Fe and Chl content decreased markedly by day 2 in –Fe plants, and finally to levels of approximately 50% of normal concentrations by day 7. The concomitant decline in Fe and Chl levels is consistent with observations in previous studies on the effects of Fe deficiency in plants (Adams, Norvell et al. 2000a; Mariotti, Ercoli et al. 1996; Zaharieva, Gogorcena et al. 2004). From a physiological perspective, the close association between leaf Fe and Chl levels is concordant with earlier findings showing that as much as 80% of cellular Fe is sequestered in the chloroplasts (Smith 1984) predominantly as an Fe^{3+}

complex in the form of the iron storage ferritin protein (Harrison and Arosio 1996; Kilcoyne, Bentley et al. 2000) and that Fe starvation leads to reduction in thylakoid and grana number (Nishio, Taylor et al. 1983) as well as impaired synthesis of chlorophylls and carotenoids (Abadía and Abadía 1993; Pascal, Block et al. 1995; Terry 1980). The high correlation between leaf Fe and Chl content obtained in this study further validates the direct effect of Fe on leaf chlorophyll content (Figure 4.14). The rapid depletion of leaf Fe levels and appearance of leaf interveinal chlorosis upon subjection to Fe stress was indicative that leaf Fe reserves were limited and that early diagnosis is crucial in managing Fe nutrition in plants.

Typical sufficiency levels of Fe in healthy vegetable crops are in the range of 50–100 ppm (Maynard and Hochmuth 1997). Generally, critical leaf Fe concentrations have been taken to be 50 ppm, whereby tissue content below this level suggest a Fe-deficient state arising from inadequate supply and/or uptake of the element (Jones 1998). Experimental data (Figure 4.12) showed that mineral levels in –Fe plants remained at or just above the critical nutrient levels established for a range of vegetable crop plants. In the case of –Fe plants, since classical symptoms were distinctly visible by day 4, while drastic reduction in Fe and Chl content was significantly evident by Day 2 compared with Ctrl plants, **the critical Fe concentration for this *Brassica* sp. was deduced to be about 80 ppm.** From the correlation equation shown in Figure 4.14, $Fe = 3.34Chl + 11.43$, **a threshold Chl value of 20 $\mu\text{g}/\text{cm}^2$ was derived, below which, the plants were in Fe-deficient state.**

Figure 4.12 Fe content in Ctrl and -Fe plants as a function of time. Mean values are denoted by closed or open circles and $\pm$ SE are indicated by the error bars.

Figure 4.13 Chlorophyll content in Ctrl and -Fe plants as a function of time. Mean values are denoted by closed or open circles and $\pm$ SE are indicated by the error bars.

Figure 4.14 Relationship between Fe and Chl content.

4.3.2.2 Reflectance Spectral Features

4.3.2.2.1 REIP method

From the 1st experiment, REIP method was established for Ca stress detection. To test its generalization, it was applied on Fe stress detection. Figure 4.15 shows the $\Delta[\text{REIP}]$ and $\Delta[\text{Fe}]$ relationship. The low r^2 value indicates that REIP method is not suitable for Fe stress detection (it also implies that the Fe stress detection will not interfere Ca stress detection). Further analysis of the sample spectra is needed.

Figure 4.15 Relationship between $\Delta[\text{REIP}]$ and $\Delta[\text{Fe}]$.

4.3.2.2.2 Specific wavelengths

Figure 4.16 validates that the R_b variation was around 0.05, therefore, as stated in section 3.4.2, reflectance can be transformed into normalized inner reflectance, which is linearly correlated with the logarithm of chlorophyll (Maccioni, Agati et al. 2001).

The PLS procedure (SAS 8.2, SAS Institute, USA) (SAS Institute Inc. 2000) was used to calculate the B-matrix and VIP to find the important wavelengths whose NR' were highly correlated with $\text{Log}(\text{Chl})$. Only when both values in B-matrix and VIP are large enough then the wavelength is considered as important. According to Wold (1995), a value less than 0.8 is “small” for the VIP. Both the B-matrix (absolute value) and VIP values in Figure 4.17 are small, therefore NR' of single wavelength is not a suitable chlorophyll indicator.

Figure 4.16 Averaged reflectance of 380–480 nm (R_b).

Figure 4.17 B-matrix and VIP.

4.3.2.2.3 CIELAB color values

Rather than looking at single wavelength, the NR^I spectrum was transformed into three CIELAB color values that contained features of the whole spectrum. The relation between L^* , a^* , b^* values and Chl content are plotted in Figure 4.18. Correlation coefficients (r^2) are shown in Table 4.1.

Figure 4.18 Linear fitting lines and experimental data for the CIELAB and Log(Chl) relationship.

Table 4.1 r^2 of the fitting in Figure 4.18.

r^2	L^*	a^*	b^*
–Fe	0.90	0.36	0.90

The correlation of L^* , a^* , b^* values to Chl content in Figure 4.18 follows a log-linear law.

$$\text{Log(Chl)} = 2.18 - 0.02L^* \tag{4-4}$$

$$\text{Log(Chl)} = -3.40 - 0.11a^* \tag{4-5}$$

$$\text{Log}(\text{Chl}) = 2.41 - 0.05b^* \quad (4-6)$$

L^* , a^* , and b^* increased as Chl content decreased. Physiological basis for the significant increase in L^* and b^* values is due to the breakdown of chlorophyll, which leads to bright yellow leaves in –Fe plants (refer to Figure 2.5). Therefore L^* and b^* were used for Chl prediction in –Fe plants because of their high correlation. In Figure 4.19, the predicted Chl content using L^* and b^* showed very high correlation with the measured Chl content extracted from leaf using conventional method. The CIELAB method was found to provide better correlation over a broader range of Chl content than other previously reported spectral indices. Adams, Norvell et al. (2000a) showed moderate agreement between ‘Yellowness index’ (YI), which measures the concavity-convexity of the reflectance spectrum near the midpoint wavelength between 550 nm and 670 nm, to total chlorophyll at concentrations less than 25 $\mu\text{g}/\text{cm}^2$ while insensitive to concentrations above 25 $\mu\text{g}/\text{cm}^2$. In fact, YI itself was not a good predictor of Fe deficiency—being correct only 30% of the time (Adams, Norvell et al. 2000b). Our data shows good correlation of L^* and b^* values to Chl up to 35 $\mu\text{g}/\text{cm}^2$. As mentioned in section 4.3.2.1.2, when the Chl concentration < 20 $\mu\text{g}/\text{cm}^2$, plants are in Fe-deficient state. Refer to Figure 4.13, –Fe plants were in Fe-deficient state by day 2. **From equation (4-4) and (4-6), when $\text{Chl} < 20 \mu\text{g}/\text{cm}^2$, $L^* > 44$, $b^* > 22$, plants were in Fe deficient state.** Therefore, using L^* and b^* Fe deficiency can be detected as early as day 2 while visual symptoms did not appear till day 4.

Figure 4.19 Correlation between predicted and measured Chl content in –Fe plants predicted using L^* and b^* .

4.3.3 P Stress Detection

4.3.3.1 Morphological and Physiological Effects of P Stress

No obvious decline in growth or visual deficiency symptoms were observed in –P plants compared to Ctrl plants. Plant physiological responses were also monitored in terms of elemental and chlorophyll content. Leaf P content in –P plants decreased markedly to about 3,000 ppm within the first 72 hours of withholding $\text{PO}_4\text{-P}$ in the nutrient solution and declined gradually to a final concentration not less than 2,500 ppm till day 7; while in Ctrl plants, the leaf P content kept in the range of 4,500–5,250 ppm throughout the experiment (Figure 4.20). On the other hand, Chl content increased in both –P and Ctrl plants, although the increase in –P plants was slightly lower than in Ctrl plants (Figure 4.21). The Chl content ranged between 20–35 $\mu\text{g}/\text{cm}^2$. Clearly, subjecting plants to P

stress over a 7-day period had no adverse impact on leaf Chl content and there was no correlation between leaf P concentration and Chl content (Figure 4.22).

Typical sufficiency levels of P in healthy vegetable crops are in the range of 3,000–6,000 ppm (Maynard and Hochmuth 1997). Generally, critical leaf P concentrations have been taken to be 2,500 ppm whereby tissue content below these levels suggests a P-deficient state arising from inadequate supply and/or uptake of the element (Jones 1998). Our data showed that mineral levels in –P plants remained at or just above the general critical nutrient levels established for a range of vegetable crop plants. The lack of visible symptoms of deficiency in –P plants and the presence of leaf P above the critical concentration by day 7 strongly indicated that the plants had not entered into the P-deficient state in this experiment. This might be attributed to surplus P stored in vacuoles being released to sustain cytoplasmic P concentrations to maintain key enzymatic and metabolic activities at optimal levels under P-deficit conditions (Lauer, Blevins et al. 1989; Lee, Ratcliffe et al. 1990; Lee and Ratcliffe 1993; Mimura, Dietz et al. 1990). Longer P deprive time might be required to manifest morphological and physiological aberrations.

Figure 4.20 P content in Ctrl and -P plants as a function of time. Mean values are denoted by closed or open circles and $\pm$ SE are indicated by the error bars.

Figure 4.21 Chlorophyll content in Ctrl and -P plants as a function of time. Mean values are denoted by closed or open circles and $\pm$ SE are indicated by the error bars.

Figure 4.22 Relationship between P and Chl content.

4.3.3.2 Reflectance Spectral Features

4.3.3.2.1 REIP method

The REIP method was applied on P stress detection. Figure 4.23 shows the $\Delta[\text{REIP}]$ and $\Delta[\text{P}]$ relationship. The low r^2 value indicates that the method is not suitable for P stress detection.

Figure 4.23 Relationship between Δ[REIP] and Δ[P].

4.3.3.2.2 CIELAB method

As the Fe-stressed plant spectra, the R_b values of P-stressed plant spectra were around 0.05 (Figure 4.24). And the small values of B-matrix (absolute value) and VIP show that NR^I of single wavelength is not a suitable chlorophyll indicator (Figure 4.25). Therefore, the CIELAB method established for Fe stress detection in the 2nd experiment was also applied on P stress detection.

Figure 4.24 Averaged reflectance of 380–480 nm (R_b).

Figure 4.25 B-matrix and VIP.

The relation between L^* , a^* , b^* values and Chl content are plotted in Figure 4.26. Correlation coefficients (r^2) are shown in Table 4.2. In the case of –P plants, leaf Chl content varied over the range of 25–32 $\mu\text{g}/\text{cm}^2$ (Figure 4.21) while L^* , a^* , and b^* values remained relatively constant. This result is similar with the observations of Adams, Norvell et al. (2000a) of the insensitivity of the ‘Yellowness Index’ to chlorophyll content above 25 $\mu\text{g}/\text{cm}^2$. This phenomena was attributed to the constancy of the reflectance spectra between 570–650 nm at high chlorophyll concentrations above 25 $\mu\text{g}/\text{cm}^2$ (Adams, Philpot et al. 1999). As discussed in section 4.3.3.1, it is likely that –P plants had not entered into a deficiency state and hence, no discernible spectral changes would be expected. This corresponds to the nutrient stress analyzer results, that all three methods—REIP, specific wavelength and CIELAB show no P deficiency in –P plants.

Figure 4.26 Linear fitting lines and experimental data for the CIELAB and Log(Chl) relationship.

Table 4.2 r^2 of the fitting in Figure 4.26.

r^2	L^*	a^*	b^*
–P	0.01	0.00	0.02

4.4 Conclusion

In the first experiment, an increase in chlorophyll content was associated with normal plant maturity and was reflected in red-shifting of the REIP position to longer wavelengths to reach steady state levels at around 714–715nm. On the other hand, anatomical changes due to breakdown in cell wall structure as a result of Ca stress might enhance the scattering of light inside the leaf, leading to increased reflectance in the near infrared region and hence blue-shifting of the REIP position to shorter wavelengths. The opposing directions of REIP shifts attributed to concomitant increases in chlorophyll content and leaf anatomical changes in Ca-stressed plants resulted in relatively constant REIP at 708 nm throughout the treatment period. This information provides the opportunity for nondestructive *in vivo* early detection of Ca deficiency in plants. **The criterion for Ca deficiency was set as: if the REIP position of a 24-day old plant (counting from 1 week of germination) did not reach or exceed 708 nm, plant was in Ca-deficient state.**

In the second experiment, since the REIP method used in the first experiment showed less capability for Fe stress detection, and no specific wavelengths of the normalized inner reflectance NR^I were found to be correlated with Log(Chl), a new method was proposed. The CIELAB values transformed from NR^I were shown to be able to provide pre-visual detection of Fe stress. This method is particularly effective for early detection of stress

where the nutrient elements have a direct influence on the levels of leaf pigments. Fe is a component of many redox enzymes and is required for the synthesis of the green pigment, chlorophyll. The direct effect of Fe on chlorophyll levels in the leaves resulted in rapid changes of L^* and b^* values, which can be used to pre-visual detection of Fe deficiency after 2 days exposure to $-Fe$ media. **The criterion for Fe deficiency was set as: if $L^* > 44$ and $b^* > 22$, plant was in Fe-deficient state.**

In the third experiment, no morphological deficiency symptom appeared in the $-P$ plants throughout the experiment. No difference was found in Chl content between $-P$ and Ctrl plants. Although there was marked decrease in leaf P content, level did not fall below critical threshold level when P supply was withdrawn within the 7-day period. This corresponds to the nutrient stress analyzer results, that all three methods—REIP, specific wavelength and CIELAB show no P deficiency in $-P$ plants. Longer exposure to $-P$ deficit conditions would be needed before plants enter into P-stressed state.

The diversity of plant nutrients might cause some nutrients to show very weak signs of deficiency or signs mixed up with other nutrients. Hence, in order to get better stress indicators, the green fluorescent protein as markers was introduced in the following chapter.

Chapter 5 'Indicator Plant' for Drought Stress Detection

This work aims to develop non-invasive spectroscopic detection of plant drought stress for field applications. Transgenic 'Indicator Plants' comprising green fluorescent protein were regenerated for concomitant use with optical methods to allow early detection of water-deficit conditions in plants.

5.1 Introduction

Currently, several biochemical and biophysical approaches have been used to assess the degree of abiotic stress in plants. Biochemical and molecular alterations are usually the first detectable and quantifiable stress responses of plants. However, analyses of biochemical indicators of stress involve destructive tissue sampling and are costly, time consuming and laborious to perform. Such approaches, thus, have not been extensively applied on a field scale (Gloser and Glaser 2001). On the other hand, monitoring biophysical parameters in relation to stress offers a more favorable approach. Thermography, chlorophyll fluorescence analysis and transmission/reflectance imaging have been used to monitor water status, surface temperature, photosynthetic efficiency and structural changes in plants for early detection of environmental stress responses (Chaerle and Straeten 2000). These non-destructive methods are more deployable at the field scale and can provide instantaneous and real-time information for objective decision-making in crop management. The problem is that in most of cases these methods are not

as accurate as biochemical methods. Therefore, new methods that can combine the advantages of biochemical and biophysical methods are required.

The study of plant molecular responses to dehydration has primarily been based on the *Arabidopsis thaliana* model system (Bartels and Souer 2003). Yamaguchi-Shinozaki, Koizumi et al. (1992) identified nine 'Responsive to Desiccation' (*RD*) genes in *Arabidopsis thaliana* and the elucidation of their corresponding cDNA sequences suggested that these genes encode membrane-associated proteins and thiol proteases. It has been found that the drought-responsive cysteine proteinases, *RD19* and *RD21*, are sensitive to changes in osmotic potential. The expression of these proteinases has been linked to the degradation of polypeptides or vegetative storage proteins to increase protein turnover rates for osmotic adjustment during times of stress (Guerrero, Jones et al. 1990; Koizumi, Yamaguchi-Shinozaki et al. 1993). Interestingly, these genes are not responsive to cold or heat stress nor transcriptionally induced by the plant hormones, abscisic acid (ABA) and gibberellin (Koizumi, Yamaguchi-Shinozaki et al. 1993). However, both were highly responsive and transcriptionally active during drought and severe salinity stress. Since ABA-mediated pathways is associated with a plethora of responses to many environmental stresses, the ABA-independent pathways that mediate induction of *RD19* and *RD21* gene expression makes the promoters of these genes useful for driving specific responses to dehydration and high salt stress (Shinozaki and Yamaguchi-Shinozaki 2000).

Enhance green fluorescent protein (EGFP) is a relatively low molecular weight protein with unique light-emitting properties, requiring only molecular oxygen and an excitation blue light source (excitation maximum at 488nm) to emit visible green fluorescence (emission maximum at 509nm). *In vivo* autocatalytic formation of the chromophore

through intramolecular polypeptide cyclization and oxidation, together with its relative resistance to photobleaching makes EGFP a desirable fluorescent tag to elucidate biological processes. Thus, this versatile reporter can be used for non-invasive and convenient monitoring of plant responses to environmental stimuli in real-time.

The aim is to develop a dual-component smart sensor system to supply critical, first hand information on plant water status with rapid feedback to an online control system for 'needs-based' application of water before onset of visible stress-induced phenotypic changes. As an initial test bed at laboratory scale, transgenic 'Indicator Plants' were developed, harbouring a chimaeric construct comprising a drought-responsive cysteine proteinase promoter fused upstream to the EGFP marker gene. These plants form the biological component of the sensor system and were expected to emit green fluorescence in response to drought stress. The fluorescence signal was detected by the prototype fiber optic spectroscopic analyzer, which is the second component of the system that allows for non-invasive field monitoring of crop water-status in real-time.

5.2 Materials and Methods

To give a vivid idea of the experimental methodology, Figure 5.1 shows the experimental flow chart.

Figure 5.1 Experimental flow chart.

5.2.1 Plant Materials

Arabidopsis thaliana (*A. thaliana*) ecotype *Columbia* (Col-O) and *Petunia hybrida* (*P. hybrida*) were grown *in vitro* on multiplication media (7g/l plant agar supplemented with 1× Murashige and Skoog medium, 30g/l Sucrose, 500mg/l casein hydrolysate, 0.04mg/ml ascorbic acid and 0.1% plant preservative mixture) under continuous illumination of approximately 2,500 luxes for 12 h at 22°C each day (Liew 1994). Leaves were harvested after 4–5 weeks. Transgenic *P. hybrida* plantlets were also grown under the same *in vitro* conditions as the wild type.

5.2.2 Isolation and Sub-Cloning of Drought Responsive Promoter (rd21A)

Genomic DNA of *A. thaliana* was extracted and the drought responsive promoter was amplified using the expand high fidelity polymerase chain reaction (PCR) system (Roche, Switzerland). Primers were designed, based on the sequence of the *rd21A* 5'flanking region ((Koizumi, Yamaguchi-Shinozaki et al. 1993), GenBank Accession No. D13043), to amplify a 1,131 bp (base pair) fragment, upstream of the translation start site. The PCR product was digested with *Xho* I and *Cla* I and then ligated into an in-house cloning vector, pChing, at the position immediately upstream of an EGFP cDNA sequence. The resulting recombinant plasmid designated pJenE21 was then transformed into competent *Escherichia coli* DH5α cells. The cloned *rd21A* promoter was verified by DNA sequencing. Subsequently, a 2,611bp *Xho* I-*Eco* RI fragment carrying the *rd21A* promoter-EGFP-3' terminator region was isolated from pJenE21 and sub-cloned into the same sites on the multiple cloning cassette of an in-house plant binary vector, pW214. In this way, the *rd21A* promoter-EGFP-3' terminator fragment was placed downstream of

the kanamycin selectable marker in the T-region between the right and left border sequences of the resulting binary vector, pW214-E21.

5.2.3 *P. hybrida* Transformation and Transformants Regeneration

The chimaeric construct was introduced into the nuclear genome of *P. hybrida* via *Agrobacterium*-mediated gene transfer. *P. hybrida* transformation was initiated by immersing young leaf disk in a liquid culture of transformed *Agrobacterium tumefaciens* harbouring pW214-E21 for 10 minutes. The leaf explants were then blotted dry and placed on regeneration medium for 2 to 3 days under low light conditions. After this co-cultivation period, the leaf explants were transferred to regeneration medium containing cefotaxime to eliminate *Agrobacterium*. Subsequently, kanamycin selection pressure was applied on the leaf explants for three to four weeks and then transferred onto regeneration medium for a further four to five weeks for shoot and root initiation. A total of forty independent transformants that survived *in vitro* selection were obtained.

5.2.4 Molecular and Expression Characterization of E21 Transformants

E21 transformants were analyzed for stable integration of intact *rd21A* promoter-EGFP-3' terminator gene construct into the plant nuclear genome. The genomic DNA was extracted and a chimaeric fragment was amplified using primers against nucleotide position -35 of the *rd21A* promoter and the 3' end of EGFP cDNA.

Prior to imposing drought stress, 4-week-old *in-vitro* grown wild type and *E21* transformants were gently removed from the culture vessels and the roots thoroughly

rinsed to remove any residual agar. These plants were then allowed to dehydrate in air on a piece of paper towel at 23°C. Plant tissues (200 mg) were harvested at 0 h, 1 h, 2 h, 4 h and 24 h time points for isolation of total RNA using the NucleoSpin RNA Plant Kit (Macherey-Nagel, Germany). The final total RNA preparation was quantitated by UV absorbance at 260 nm and quality verified by agarose gel analysis. The total RNA samples were then used as templates for reverse transcription-PCR (RT-PCR) detection of EGFP transcripts in response to dehydration. As an internal control to demonstrate the absence of contaminating genomic DNA in the total RNA preparations, RT-PCR was also carried out using primer pairs that span across two exons of the *Petunia* actin gene (GenBank Accession No CV301219). The presence of contaminating genomic DNA would result in amplification of a 550 bp actin fragment that contains a full intron in between the two exons. Desired amplification from reverse transcribed actin mRNA would result only in a 219 bp PCR product.

EGFP fluorescence expression following induction of drought stress was monitored at 0 h, 1 h, 2 h, 4 h and 24 h time points respectively. Excited by a blue laser (LH-M100C-1, Nikon, Japan), a wild type plant and a transformant were simultaneously monitored under a microscope comprising a specific filter set (SMZ1500 stereoscopic zoom microscope, Nikon, Japan) and a digital camera (DS-SMc, Nikon, Japan). Images were captured by the camera, and the EGFP emission spectra were captured concomitantly by a spectrometer (USB2000, OceanOptics, USA). Figure 5.2a shows the microscope used for data observation and capturing. For field application, a portable system was shown in the Figure 5.2b. Similar to the setup shown in Figure 3.1b, except a filter was added in front of the spectrometer and the light source was changed into a blue laser diode.

Figure 5.2 (a) Microscope for image and fluorescence emission spectra capturing. The area marked with dash line circle is where to connect the camera and spectrometer. (b) Portable drought stress analyzer for field application.

5.3 Results and Discussion

5.3.1 Isolation of Drought Responsive Promoter, *rd21A*

The 5' flanking regions of the drought-responsive cysteine proteinase genes, *RD19* and *RD21*, from *A. thaliana* contain two conserve sequences which were thought to be *cis*-acting elements regulating the expression of these genes (Koizumi, Yamaguchi-Shinozaki et al. 1993). However, the functional domains of these drought-responsive promoters have not been fully defined. By comparing homologies between the 5' flanking regions of *RD19* and *RD21*, the conserved sequences between these two genes were found located within 1,480 bp and 1,131 bp fragments of the upstream regions, respectively. Hence, the functional portion of the *RD21* was deduced to be located within the 1,131 bp upstream fragment. DNA sequencing of one of our *RD21* bacterial clones showed 100% homology to the published sequence.

Regenerated *E21* plant transformants were screened by PCR for the presence of intact *rd21A* promoter-EGFP cDNA transcriptional fusions in the nuclear genome (Figure 5.3). The expected 800 bp amplified product was observed in thirty five out of forty putative transformants screened.

Figure 5.3 PCR confirmation of two E21 transformants (A and B) carrying the intact rd21a-EGFP chimaeric construct. Amplification from the cognate plasmid vector (+ve Con) and genomic DNA from leaves of wild-type *Petunia* (wt) are shown.
M: 100 bp ladder.

5.3.2 EGFP Expression under Desiccation Stress

5.3.2.1 PCR Result

The quality of total RNA obtained from plant tissues declined with increasing time from induction of desiccation stress (Figure 5.4). The ribosomal bands in the 24 h time point sample were less distinct on an ethidium bromide-stained agarose gel, significant background smearing and presence of smaller size RNAs were seen. These indicated that, as the desiccation stress went on, cellular degradation increased.

Figure 5.4 Qualitative analysis of total RNA isolated from a drought-stressed *Petunia* transformant at indicated time points (0 h, 1 h, 2 h and 24 h).

λ: Lambda *Hind III* marker.

RT-PCR of actin mRNA transcripts was carried out to demonstrate the absence of contaminating genomic DNA in the total RNA preparations (Figure 5.5). The presence of only the 219 bp and the absence of 550 bp band showed that the amplified actin band arose from correctly reverse transcribed actin mRNA and contaminating genomic DNA was not present in any of our total RNA samples. Analysis of the level of actin transcripts also provided endogenous internal references of gene expression over the period of stress. It has been reported that in times of stress, plants undergo transcriptional reprogramming, resulting in a decrease in transcription of 'house-keeping' genes, and upregulation of other genes (Volkov, Panchuk et al. 2003). Figure 5.5 clearly showed that the level of actin mRNA transcripts isolated from *Petunia* significantly reduced at the 24 h time point. It is difficult to conclude whether this was due to downregulation of actin gene expression *per se* or to the decline in RNA integrity attributed to increased severity of desiccation damage.

A preliminary trial (data not shown) investigating the induction EGFP mRNA transcripts 24h after induction of stress showed that the *rd21A* promoter in the transformants was responsive to dehydration, salt and sucrose stress. Significant levels of EGFP mRNA were detected by RT-PCR but not from wild type plants. This suggested that changes in the turgor pressure due to osmotic stress arising from water deficit, high salt or sugar in the root environment could trigger EGFP transcription from the drought-responsive promoter (Yamaguchi-Shinozaki and Shinozaki 1993). As shown in Figure 5.5, weak basal levels of EGFP transcripts prior to dehydration were detected and a steady increase in levels up to maximum at 2 h following onset of desiccation. Despite the evident degradation of total RNA after 24 h desiccation (Figure 5.4), a weak amplified EGFP band was still observed, alluding to the stability of the EGFP transcripts.

Our results compared very well with previous reports that the *RD21* gene in *A. thaliana* was induced by dehydration and high salt environmental stress conditions, whereby the cognate mRNA transcripts were detected after 1.5 h and accumulated steadily up to 24 h (Yamaguchi-Shinozaki, Koizumi et al. 1992). This pattern of promoter activity in a heterologous environment was not surprising, as stress responses in plants are thought to be mediated via universal signaling pathways (Harper, Mabon et al. 1999; Hu and Cheng 1995; Naylor 1999).

Figure 5.5 EGFP (E) and Actin (A) mRNA transcript induction profile between drought stressed wild type (a) and transformed (b) Petunia at indicated time points 0 h, 1 h, 2 h, and 24 h. Con: control, M: 100 bp ladder.

5.3.2.2 Fluorescence Image

Under the microscope, red chlorophyll autofluorescence was clearly observed in wild type plants and unstressed *E21* transformants (Figure 5.6). As dehydration stress progressed, green fluorescence was gradually seen accumulating, only from 2h onward, in the stems, leaf mid-veins and leaf tips of a stressed *E21* transformant, and peaking at 24h (Figure 5.6d-f). The ability of EGFP to absorb blue light and emit green light is dependent on cyclization and oxidation of the T-Y-G tripeptide for chromophore formation within its β -barrel structure. Following protein synthesis, the time taken for chromophore maturation in wild type GFP and S65T mutant GFP has been reported to be about 2–4 h and 0.45 h, respectively (Heim, Prasher et al. 1994; Heim and Tsien 1996). Sheen, Hwang et al. (1995) reported that GFP fluorescence could be visualized in maize mesophyll protoplast only 8 h after transfection. Verkhusha, Akovbian et al. (2001) showed that EGFP maturation required about 1.5–2 h. Thus, the kinetics of post-translational chromophore formation and maturation is a key property governing the ability to report any subtle and rapid response following induction of GFP gene expression (Cubitt, Heim et al. 1995).

However, increasing background green fluorescence was also observed at the chlorotic and necrotic sites of the wild type plants (Figure 5.6a-c). This may be due to the refractile nature and light scattering properties of plant cell walls (Haseloff and Amos 1995), especially with the progression of degenerative cellular and tissue injuries leading to structural distortion in severely stressed plants (Dendron Resource Surveys Inc. 1997). Furthermore, endogenous autofluorescence around 520–560 nm has also been attributed to plant constituents like flavins and lignins and the typical excitation wavelengths of these molecules coincides with that of EGFP at 488 nm (Chappelle and Williams 1987; Knight and Billinton 2001).

Figure 5.6 Fluorescence images of drought stressed wild type (a–c) and E21 transformed plants (d–f) at 2 h, 4 h and 24 h time points.

Strong but randomly localized EGFP expression in the vascular tissues of stems in the plant transformant was also observed (Figure 5.7d–f). Background fluorescence from endogenous cellular components was less problematic in the stems (Figure 5.7a–c), thus making it easier to distinguish EGFP fluorescence.

Figure 5.7 Fluorescence stereoscope images of stems of drought-stressed wild type (a–c) and E21 transformed plants (d–f) at 2 h, 4 h and 24 h time points.

5.3.2.3 Fluorescence Spectra

EGFP emission signals from the desiccation-stressed *E21* transformant was detected at 0 h, 1 h, 2 h, 4 h and 24 h time points (Figure 5.8) using a spectrometer, the amplitude of the signal increased gradually to maximum at 24 h, whereas the spectrum obtained from wild type plants remained at baseline levels throughout the desiccation period (data not shown). The gradual increase in signal intensity with desiccation time coincided well with the observation of increased EGFP emission from the fluorescence images. However, the peak emission wavelength was observed to be around 530 nm instead of the expected 509 nm emission maxima. As mentioned in section 5.3.2.2, autofluorescence around 520–560 nm from plant constituents like flavins and lignins may partially overlapped with GFP signal, therefore the fluorescence maximum peak was red-shifted.

Figure 5.8 EGFP fluorescence spectra from E21 transformant following onset of desiccation stress, (a) at 0 h, 1 h, 2 h, 4 h and 24 h; (b) subtracted 0 h signal from the 1 h, 2 h, 4 h and 24 h signal. The 509 nm position is indicated by the vertical line.

5.4 Conclusion

Transgenic *Petunia* plants carrying drought-responsive promoter-linked EGFP genes were developed as bioindicators of drought-stress in plants. The temporal and spatial patterns of stress-induced EGFP expression under the control of the ABA-independent drought-responsive promoter *rd21A* in the transgenic *Petunia* were reported. Spectroscopic detection of EGFP expression was evaluated against classical biochemical and molecular analyses at the transcriptional level. At the whole plant level, the induction of the fluorescent marker gene by desiccation stress was also monitored under the microscope.

The results showed that while an increase in EGFP mRNA transcripts could be detected after 1 h desiccation stress, green fluorescence emission above endogenous background

could only be observed by microscope after 2 h. The spatial distribution of EGFP fluorescence was also found to be non-uniform and sporadically localized in certain parts of stems and leaves, particularly the vascular tissues. Therefore, the utility of EGFP as a sensitive and rapid reporter of desiccation stress will be compromised by the presence of endogenous background fluorescence that overlaps with the EGFP emission wavelength, chromophore maturation and its non-uniform spatial distribution pattern when fluorescence detection methods are employed. Addressing these biological issues and further fine-tuning of the detection methodology is required to make this bioindicator-cum-optical detection approach a turnkey technology for monitoring drought stress under field situations.

Both the nutrient stress analyzer and the 'indicator plant' detect plant responses to specific parameters. To study plant response to its growth environment in an overall extent, photosynthesis system will be an attractive area to explore. Since the quantity of CO₂ gas absorbed by plant is a standard to evaluate photosynthesis process, in the following chapter, systems developed to monitor ambient CO₂ content will be introduced.

Chapter 6 Fiber Cavity Ring Down Spectroscopy

Various kinds of fiber cavity ring down systems were set up and modified for gas detection purpose. The high intrinsic loss fiber bragg grating (FBG) ring down cavity system was first modified into a passive coupler fiber ring down cavity system with a long period grating (LPG) as sensing portion, and finally to an active coupler fiber ring down cavity system for CO₂ gas detection.

6.1 Introduction

Because of the overwhelming greenhouse effect, the demand of a green earth is increasing. Growing plants is one of the major remedies for a green earth, as plants consume CO₂, convert it into their food through photosynthesis process. Therefore, it becomes necessary to study the relation between plants and CO₂. On the other hand, from farmers' point of view, it is important to know how plants response to the elevated CO₂ environment. It is also possible to monitor plant health by monitoring the CO₂ content of its growing environment, because if plant is in an unhealthy state, its photosynthesis process might be abnormal, less or more CO₂ gas will be absorbed, and further stress might occurred (Hamer 2003; Keutgen and Chen 2001; Larbi, Abadía et al. 2004; Pal, Karthikeyapandian et al. 2004; Pieters, Paul et al. 2001; Singh and Blanke 2000). Of all these studies, the detection of CO₂ gas is one of the key issues. Several systems have been developed for this purpose, e.g. photosynthetic measuring system (LI-6400, LI-COR, Inc.,

USA), CO₂ infrared gas analyzer (PP Systems, UK). These systems use expensive mid-IR laser sources and can only perform localized measurement.

Actually, not just for CO₂ gas detection, gas-phase absorption spectrometric measurements are of interest in a variety of applications including pollution monitoring and control, remote sensing, trace analysis of high-purity gases such as those used in semiconductor fabrication, combustion studies, and explosive agent detection. While traditional absorption spectrometry has been applied to all of the above measurements situations, its sensitivity has frequently not been adequate for the task. And although there have been significant advances in the development of mid-IR laser sources, near-IR (1–2 μm) region is still the best option for remote and multipoint sensing of hazardous or environmentally important gases because of the ready availability of fiber optic components from the telecom industry. However, a key issue for the applications of near-IR systems is the development of high sensitivity techniques for the relatively weak overtone absorption lines that are typically three or more orders of magnitude weaker than the fundamental lines in the mid-IR.

The newly developed fiber cavity ring down spectroscopy holds the promise to solve to above problems. The aim is to develop a sensitive, remote sensing capability and low cost gas sensing system. The new fiber cavity ring down spectroscopy was explored and systems were set up and modified for CO₂ gas detection purpose.

6.2 Materials and Methods

6.2.1 Fiber Bragg Grating (FBG) Fiber Cavity

Figure 6.1 shows the FBG fiber cavity ring down system. The FBGs were used in place of the mirrors in a traditional ring down cavity. The FBGs, made in hydrogen-loaded SMF with a chirped phase mask, were 1 cm long, high reflective (99%–99.9%) with a bandwidth of ≈ 0.2 nm. Light from the CW laser (Model 8164A, Agilent, USA) passed through the isolator (ISO, WTD, China) that can avoid light reflected back to the laser, was modulated by acousto-optic modulator (AOM, Brimrose, USA) with 40 kHz, coupled into the cavity by FBG1, went through 50 m long single mode fiber (WTD, China), then coupled out of the cavity from FBG2 into the photodetector (PD, Model 1811 IR DC-125MHz low noise photoreceiver, New Focus, USA). The FBGs and SMF were connected using an arc fusion splicer (Fujikura, Japan).

Figure 6.1 FBG fiber cavity ring down system.

6.2.2 Coupler Fiber Cavity

6.2.2.1 Passive Cavity

Figure 6.2 shows the passive coupler fiber cavity ring down system, similar to the FBG fiber cavity ring down system, except that the FBGs were replaced with two couplers (WTD, China), each with a coupling ratio of 1:99. A long period grating (LPG) was used as sensing part inside the cavity. A gain tunable erbium-doped fiber amplifier (EDFA) was added after the AOM to enhance the light coupled into the cavity (no ISO was used after the laser because the EDFA has a built in ISO). A circulator and FBG cascaded in pair were used as a filter set to filter out the amplified spontaneous emission (ASE) noise from the EDFA. Figure 6.3 shows a typical ASE spectrum after passing through the filter set. Only the wavelength of interest was reflected by the FBG and passed through coupler1 into the cavity.

Figure 6.2 Passive coupler fiber cavity ring down system.

Figure 6.3 ASE of EDFA pass through circulator-FBG filter set.

6.2.2.2 Active Cavity

Figure 6.4 shows the active coupler fiber cavity ring down system. Similar to the passive coupler fiber cavity ring down system, but a 9-cm long open path fiber bench (Zugo, USA) was used as sensing part inside the cavity. The insertion loss of the fiber bench is ~ 4 dB. To overcome this loss and mimic the high finesse, low loss cavities used in conventional CRDS systems, a gain tunable EDFA and a circulator-FBG filter set were added into the cavity which made it an active cavity. The ring down time (and hence the sensitivity) can be adjusted by changing the EDFA's gain.

Figure 6.4 Active coupler fiber cavity ring down system.

6.3 Results and Discussion

6.3.1 FBG Fiber Cavity

Figure 6.5 shows the ring down signal of FBG cavity system. The averaged ring down time is 1.46 μs (200 ring down events were recorded and 200 ring down time were obtained and averaged), recall equation $\Gamma = 4.34t_r/\tau$ (2-18), the calculated round trip loss is 6.87 dB. According to the cavity composition, the round trip loss is

$$\Gamma = 2L_{\text{splicing}} + 2L_{\text{FBG}} + L_{\text{SMF}} \quad (6-1)$$

where L_{splicing} is the loss of the splicing points between the FBGs and SMF, L_{FBG} is the loss of the FBG, L_{SMF} is the absorption and scattering loss of the SMF. The loss of each splicing point is less than 0.1 dB using an arc fusion splicer, the absorption and scattering loss of the SMF is 0.35% (0.007%/meter, data provided by WTD, China), which is 0.015 dB, in 50-m length. If we assume L_{splicing} equals to 0.1 dB, the L_{FBG} will be around 3.3 dB.

Figure 6.5 Ring down signal of FBG fiber cavity.

Normally, L_{FBG} comprised of grey loss and cladding-mode loss (Wang and Po 2003), given as:

$$L_{FBG} = L_{grey} + L_{cladding} \quad (6-2)$$

Grey loss, also known as the background loss, includes absorption, scattering and transition losses in the hydrogen-loaded FBG due to ultraviolet (UV) illumination during the grating fabrication process. For 1-cm long FBG, this loss can be as much as 0.3 dB (Johlen, Knappe et al. 1999). Cladding-mode loss is related to the fiber structure as well as the fabrication, and dependent on wavelength. In principle, the reflection spectrum of a fiber grating is complementary to its transmission spectrum: What is not transmitted is reflected. However, a typical FBG suffers additional transmission loss for which there is no corresponding reflected light. This loss, typically at a level of less than 0.3 dB

(Ouellette Jan 2001), is caused by light being reflected into the cladding modes of the fiber. According to equation (6-2), $L_{FBG}=0.6$ dB, it is nowhere near the 3.3 dB!

The reason for this high loss might be: the operation wavelength was not in the FBG's high reflective band region. Because of the narrow bandwidth (~ 0.2 nm) of the FBG's high reflective region and the FBG's sensitivity to temperature, it is very hard to control the two FBGs' high reflective region overlap to each other. Therefore, it is possible that the overlap region of the FBGs was not at the highest reflective region. Figure 6.6 shows the reflective band from FBG1, by just shifting 0.1 nm aside the edge of the highest reflective region (indicated by two arrows), the loss increased by more than 1 dB.

Figure 6.6 Reflectance of FBG1.

In the future, the cavity intrinsic loss can be reduced from the following aspects. First, the FBG's grey loss can be reduced using deuterium-loaded instead of hydrogen-loaded FBGs. In hydrogen-loaded FBG, a weak, broad overtone absorption of the by-product, Ge-OH, at 1.41 μm extends to 1.55 μm . This absorption loss is approximately 0.1–0.2 dB/cm within the FBG (Johlen, Knappe et al. 1999). While in deuterium-loaded FBG, the by-product of UV exposure is Ge-OD, which absorbs at 1.9 μm , giving rise to a much smaller absorption at 1.55 μm (Kashyap 1999). Second, improve the FBG fabrication process, carefully control the UV light power and the exposure time to reduce physical damage of the grating and increase the high reflective bandwidth of the FBGs, and if it is possible, control the working temperature of the FBGs to make it easy for band overlapping control.

Because of the high intrinsic loss of the current FBG cavity and the limitation of the operation wavelength—it has to be within both FBGs' high reflective band, optical coupler was used, since it has no such critical requirement for the operation wavelength.

6.3.2 Coupler Fiber Cavity

6.3.2.1 Passive Cavity

Figure 6.7 shows the fitted exponential ring down curves of the passive coupler fiber cavity system. When the LPG was in air, ring down time was $\sim 1.26 \mu\text{s}$, calculated round trip loss was $\sim 0.82 \text{ dB}$; when the LPG was immersed into pure ethanol solution, ring down time was $\sim 2.46 \mu\text{s}$, calculated round trip loss was $\sim 0.42 \text{ dB}$, therefore, the loss difference between in air and in ethanol was $\sim 0.40 \text{ dB}$. Compared with the value tested

using direct absorption measurement, 0.37 dB, the values are quite similar. This confirmed that the coupler fiber ring down cavity method really worked.

Figure 6.7 Fitted exponential ring down curves of LPG in air and in pure ethanol solution.

To detect CO₂ weak transition around 1550 nm (Figure 6.8 shows the CO₂ gas absorption line strength at 296K (Rothman, Jacquemart et al. 2005)), the sensing part has to be modified to response to or absorb CO₂. Tapered fiber, etched fiber, and fiber bench had been considered and tried (result not shown). Although has low insertion loss, tapered fiber did not response to CO₂, this might because of the weak transition. Both etched fiber and fiber bench had high insertion loss not suitable for passive cavity. Therefore an active cavity was developed and tested with a fiber bench inside the cavity as an open path gas cell.

Figure 6.8 CO₂ gas absorption line strength at 296K.
The inserted figure zoomed in the range from 1545–1580 nm.

6.3.2.2 Active Cavity

The system was used to detect CO₂ transition in wavelength 1550.5802nm (6449.199 cm⁻¹). It was chosen because of the limitation of the circulator-FBG filter set's operation wavelength (1550.50–1550.65 nm). In this position, CO₂ has a transition with a very weak line strength $S = 8.06 \times 10^{-27}$ cm⁻¹/(molecule cm⁻²). In the experiment, the gas chamber pressure changed from 0 to 35 psi, therefore, γ changed from about 0.067–0.15 cm⁻¹/atm (Rothman, Jacquemart et al. 2005). Recall Beer-Lambert Law, $I = I_0 \exp(-\sigma(\nu)LN)$ (2-13), $\sigma(\nu) = S(\nu)/(\pi\gamma(\nu))$ (2-14), the loss produced in an open path cell of length l is $4.34\sigma Nl$ dB. Assume $\gamma = 0.1$ cm⁻¹/atm, $N = 10\%$, the additional loss $\Delta\Gamma$ is therefore

equals to 2.48×10^{-6} dB, for a 9 cm long fiber bench. From equation $\Gamma = 4.34 t_r / \tau$ (2-18), we can give:

$$\Delta \tau / \tau = \Delta \Gamma / \Gamma \quad (6-3)$$

Assumed that a 1% change in the ring down time can be experimentally measured, $\Delta \tau / \tau = 1\%$, then $\Gamma = 2.48 \times 10^{-4}$ dB. Therefore, for the loop, round trip time $t_r = 0.4 \mu\text{s}$, τ has to be 7 ms. The effective length is $9(\tau/t_r) = 157.5$ m.

According to Stewart, Atherton et al. (2004), the introduction of gain within a fiber cavity is necessary for achieving long ring down times whenever the insertion loss of the sensor device in the loop becomes significant (≥ 0.1 dB). The 9-cm long open path fiber bench has an insertion loss of approximately 4 dB, if a passive loop is used, for 80-m long cavity, the ring down time will be of the order of 400 ns. Hence an EDFA is introduced into the cavity to overcome this loss. While the introduction of gain within a fiber cavity presents difficulties relating to gain control, stability and non-linear effects. Stewart, Shields et al. (2004) presented a gain-clamped optical fiber loop and introduced digital bandpass filtering techniques to remove unwanted spectral components from the data arising primarily from relaxation oscillations. But still, their system showed that between adjacent ring down event, the recorded ring down time may change by as much as 30%, even with no gas present in the loop.

Figure 6.9 shows ring down time in vacuum increased as the EDFA's gain increased. When the gain was higher than a certain value, the signal became unstable. Ring down times that longer than $30 \mu\text{s}$ are not trustable, variations can go up to 100%. It is obvious that the system doesn't have enough resolution and stability to test such weak CO_2

transition in 1550.5802nm. If we test the relative stronger transition in 1572.66 nm, at this position $S = 1.82 \times 10^{-23} \text{ cm}^{-1}/(\text{molecule cm}^{-2})$, the additional loss $\Delta\Gamma$ equals to 0.006 dB. When $\Delta\tau/\tau = 1\%$, then $\Gamma = 0.6 \text{ dB}$ and τ is 3 μs , the system will be able to pick up the difference down to 1% ($\tau = 30 \mu\text{s}$).

Figure 6.9 Fitted exponential ring down curves.
Ring down time increased as EDFA gain increased.

For our system, thermal effects might cause lots of instability. First, the effective refractive index of the fiber is temperature dependent (Tamura and Nakazawa 1996; Whitenett, Stewart et al. 2004). If temperature varied, the effective refractive index varied, and so as the cavity length, though the SMF should only carry one transverse mode, mode hopping still occurred (von Lerber and Sigrist 2002a). Second, the EDFA was not operated within temperature cooling control environment, during the operation, the heating from its own circuit caused the gain varied. Third, circulator-FBG was used as

filter in our system and FBG operation wavelength is also sensitive to temperature variation. Stewart, Shields et al. (2004) also stated that the filter used in the fiber cavity should have a very narrow bandwidth, because for cavity length 10–100 m, the cavity mode spacing is in the order of tens of MHz, while the filter used in our system has the bandwidth of the order of tens of GHz, this also caused mode hopping. The mode hopping effect might be minimized by combination of wavelength modulation technique (Stewart, Atherton et al. 2004; von Lerber and Sigrist 2002a). The system of introducing gain inside ring down cavity is therefore needed further improvements for trace gas measurements.

6.4 Conclusion

The short ring down time of FBG ring down cavity system, in the order of 1.5 μ s, showed the high cavity intrinsic loss. The loss was thought to be caused by $L_{splicing}$ (loss of the splicing points between the FBGs and SMF), L_{SMF} (absorption and scattering loss of the SMF), and L_{FBG} (loss of the FBG). While $L_{splicing}$ and L_{SMF} only caused ~ 1 dB loss, most of the intrinsic loss might caused by L_{FBG} . But normally L_{FBG} is ~ 0.6 dB if working in the high reflective region. Therefore, the unexpected high loss might be because the FBGs were not working in high reflective region. It was hard to control two FBGs' high reflective region overlap to each other because of their narrow bandwidth (< 0.2 nm) and the effect of temperature variation. Therefore passive coupler fiber ring down cavity system was introduced.

LPG was used as sensing part in the passive coupler fiber ring down cavity. The trial test on putting the LPG in air and pure ethanol gave ring down time ~ 1.26 μ s and ~ 2.46 μ s

respectively. The measured loss difference of in air/ethanol (0.4 dB) is quite similar to the one measured by conventional single pass absorption method (0.37 dB). This confirmed the working capability of the coupler fiber cavity.

An active coupler fiber cavity with an open path fiber bench as sensing part was formed by adding a gain adjustable EDFA in the cavity. Although up to 30 μ s ring down time can be achieved, it was not enough sensitive to detect CO₂ gas in the weak absorption wavelength of 1550.5802 nm. The introduction of gain within a fiber cavity presents difficulties relating to gain control, stability and non-linear effects. When gain was higher than a certain value, the signal became unstable. Ring down time that longer than 30 μ s was not trustable. The instability might be caused by mode hopping due to thermal effect on fiber, EDFA and FBG filter and the broad bandwidth of the FBG filter. Further improvements were needed before the active ring down cavity can be used for trace gas measurements.

Chapter 7 Conclusions and Recommendations

7.1 Conclusions

In this thesis, three detection systems have been developed for rapid and non-destructive monitoring of plant responses to the growth environment. The first system is a nutrient stress analyzer for mineral nutrient stress detection. The second is a dual component system that comprises the optical detector and a novel transgenic indicator plant for drought stress detection. The third is a fiber optic cavity ring down spectroscopic system for carbon dioxide gas detection. The promising results obtained in this work demonstrate the potential of these systems for further development for field applications in precision agriculture and environmental monitoring.

7.1.1 Nutrient Stress Analyzer

A nutrient stress analyzer developed from off-the-shelf components was successfully tested for plant nutrient stress detection. Both laboratory and portable systems have been designed with pre-visual detection capability and added advantageous features of being non-destructive, remotely accessible and user friendly. The portable system extends the usage of the system beyond the laboratory to the field. A defined series of steps for the collection of reflectance spectrum from a plant leaf sample was established and the protocol for spectral acquisition is outlined in the Appendix. The acquired reflectance spectrum was analyzed using different spectral analysis strategies comprising the red edge inflection point (REIP), specific wavelength detection and transforming the entire

spectrum into CIELAB color values. The spectroscopic system along with the spectral analysis methodologies were used for monitoring calcium, iron and phosphorus deficiencies in *Brassica chinensis* L. var *parachinensis* (Bailey) grown under hydroponics conditions. Conventional total elemental and chlorophyll content measurements were also carried out to corroborate the physiological status of the plant against spectral features obtained from the nutrient stress analyzer.

In the calcium (Ca) deficiency tests, the total elemental Ca content in the plants was found to fall below the critical nutrient threshold level 4 days after calcium deprivation. It was observed that an increase in chlorophyll content was associated with normal plant maturity and this was reflected in red-shift of the REIP to longer wavelengths at 714–715 nm. On the other hand, for Ca-deficient plants the REIP remained constant at 708 nm throughout the test period. This is attributed to the opposite effect that progression towards maturity and calcium-induced plant cell wall breakdown has on REIP. While plant maturation led to red shifting of REIP, the breakdown of the plant cell wall promoted a countering blue-shift in Ca-stressed plants. There was no visual difference between control and Ca-stressed plants throughout the experiment time. Therefore, an *in vivo* early detection criterion for Ca deficiency is proposed as follows:

If the REIP of a 24-day old plant (counting from 1 week of germination) does not exceed 708 nm, the plant is in a Ca-deficient state.

From the iron (Fe) deficiency studies, it was shown that neither the REIP nor specific wavelength method were good indicators of iron deficiency states. However, data processing by transformation of the normalized reflectance spectra (NR^I) into CIELAB values showed significant variations in the L^* and b^* values in response to Fe deprivation.

This was attributed to the direct effect of Fe on chlorophyll levels in the leaves resulting in rapid changes in CIELAB (color) values. The following criterion for Fe deficiency is thus established:

When $L^* > 44$ and $b^* > 22$, then the plant is deemed to be in an Fe-deficient state.

It was observed that these minute spectral changes could be detected within two days of the plant being subjected to Fe stress. This is a significant finding as yellowing of the plant leaf was visually distinguishable only after 4 days of Fe deprivation.

In the Phosphorus (P) stress experiments, no morphological deficiency symptoms appeared in the P-stressed plants throughout the experiment. Both the P-stressed and control plants showed similar levels of leaf chlorophyll. Although chemical analysis revealed a decline of P content in P-stressed plants, P levels did not fall below the critical threshold level within the 7-day test period. Spectral data obtained from the nutrient stress analyzer did not show significant deviations in the REIP, specific wavelengths and CIELAB color values compared with the control plants. Hence, the spectral data corroborated well with the physiological and morphological evidence that the test plants had not entered into a phosphorus deficient state and that a much longer duration of imposed P-deprivation is likely required to elicit P deficiency.

7.1.2 'Indicator Plants'

Transgenic 'indicator plants' with a portable interrogation system have been developed and tested for drought stress detection. Transgenic *Petunia* plants carrying a drought-responsive promoter-linked EGFP gene construct were developed as bioindicators of drought-stress in plants—the plants emit green fluorescent light when they were subjected

to drought stress. Molecular and spectroscopic characterization of the drought-induced EGFP gene expression as a function of time and space was carried out. The induction of the fluorescent marker gene by desiccation stress was also monitored under a fluorescence microscope.

The results showed that an increase in EGFP mRNA transcripts could be detected 1 hour after the plant was subject to desiccation stress while green fluorescence emission above the endogenous background could be observed by fluorescence microscopy after 2 hours. Both fluorescence images and spectra showed similar results that EGFP fluorescence intensity increased as the stress time increased peaking at 24 h. In addition, the spatial distribution of EGFP fluorescence was found to be non-uniform and microscopy images showed localized patches of EGFP fluorescence in the leaves and stems. However, lower endogenous background fluorescence level was observed in stems and therefore interrogating this plant part was advocated for detection of drought-induced EGFP fluorescence signal. In summary, transgenic 'indicator plant' as a sensitive and rapid reporter of drought stress has been successfully demonstrated and its concomitant use with a portable spectroscopic system will make a great contribution towards monitoring plant water status in agriculture.

7.1.3 Fiber Cavity Ring Down System

Fiber optic cavity ring down systems have been built to detect ambient carbon dioxide gas content. Different cavities were developed and evaluated, Fiber Bragg Grating (FBG) reflector based cavity, passive coupler reflective cavity, and active coupler cavity, to detect weak absorption gas and reduce system intrinsic loss.

The short (1.5 μ s) ring down time of FBG ring down cavity system exhibited high cavity intrinsic loss. This was thought to be mainly caused by the two FBGs not operating in overlapping high reflective regions. Therefore, a passive coupler fiber ring down cavity system was developed. A Long Period Grating (LPG) was used as sensing section in the cavity. Trial tests with air and pure ethanol gave results similar to those measured by conventional single pass absorption method. However, the sensitivity was still low and hence an active cavity was developed. The LPG was replaced with an open path fiber bench as sensing part and a gain adjustable Erbium Doped Fiber Amplifier (EDFA) was introduced in the cavity to compensate for the intrinsic loss and thus gave longer ring down time. However, these introduced other side effects such as gain control and unstable signals due to mode hopping caused by thermal effect on fiber, EDFA and FBG filter and the broad bandwidth of the FBG filter. A 30 μ s stable ring down time was achieved, which could detect CO₂ gas at the weak absorption wavelength of 1572.66 nm but not at the tested 1550.5802 nm. Further improvements will be needed for the active ring down cavity to detect CO₂ gas in ~1550 nm region.

7.2 Achievements

A nutrient stress analyzer, which is portable, remote accessible, non-destructive, user friendly and provides early detection capability on the basis of a set of objective parameters, has been developed. This is the first time a system has been reported to be able to predict nutrient stress non-destructively.

A novel idea of using transgenic 'indicator plants' concomitantly with a portable spectroscopic system for plant stress detection was proposed. This is the first time to use a

plant as an ‘alarm’ for drought stress and the first time to generate transgenic drought stress inducible fluorescent plants. The bioindicator-cum-optical detection approach provides a springboard to develop other useful field monitoring applications by linking the array of available fluorescent marker genes with other stress inducible promoters.

To aid physiological studies of plant photosynthetic processes, fiber optic cavity ring down systems have been built to detect ambient carbon dioxide gas content. This is the first report describing the use of fiber optic cavity ring down system for carbon dioxide gas detection in the weak absorption near infra-red region.

Publications based on these work are shown in the last page of the thesis.

7.3 Recommendations

For the nutrient stress analyzer, further investigations are needed for the other essential nutrient elements. It is important to verify if the spectral features obtained from other nutrient deficiencies are unique to each elemental disorder. Furthermore, there is need to consider extending the experimental period for investigations of nutrients that need a longer time for the manifestation of stress symptoms. The greatest challenge is to identify diagnostic spectral signatures for essential nutrient elements that have no direct physiological influence on plant leaf color. Finally, trial tests of the system in the field needs to be carried out to verify the results of plant nutrient deficiencies obtained under laboratory conditions in this work.

The utility of EGFP as a sensitive and rapid reporter of desiccation stress could be compromised by the presence of endogenous background fluorescence that overlaps with the EGFP emission wavelength, slow chromophore maturation and the non-uniform spatial EGFP gene expression pattern. Addressing these challenging biological issues and further fine-tuning of the fluorescence detection methodology is required to make indicator plants a turnkey technology for monitoring drought stress under field situation.

The high intrinsic loss of the FBG ring down cavity system needs further investigation. Possible improvements of FBG fabrication may be achieved through the use of deuterium-loaded fiber, control UV light power and exposure time during FBG fabrication. Further improvement is needed for signal stabilization of the active coupler ring down cavity system. The combination of wavelength/frequency modulation spectroscopy (Rabinovich, Svimonishvili et al. 2002; Rabinovich, Svimonishvili et al. 2004) might be helpful.

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Appendix

Acquisition Protocol for the Nutrient Stress Analyzer

For proper and systematic use of the Nutrient Stress Analyzer for mineral nutrient deficiency, the acquisition of the reflection spectrum is described as follow:

The reflection spectrum from the leaf is collected using the modified reflectance probe with a dark sponge to support the underside of the leaf. The spectrum is recorded from the same region of the same leaf at the same time in 24 hour intervals. The spectrum collection positions (solid line circles) are located at the lower base of the leaves as shown in Figure A.1. These two points are in the location where leaf width is the widest and within the central area (marked in dash-dot lines) between leaf main vein and margin. Since a tissue element is displaced away from the leaf base as a result of production of younger tissue below it (Bernstein, Silk et al. 1993), deficient symptoms were thus expected to first appear around the basal region of the leaf (in our studies, the calcium and iron deficient symptoms did appear first from the basal region of the leaf, for other nutrient stress whose deficient symptom might appear first from leaf tip, future study is needed). This does not however, restrict the data acquisition to be at a specific location. As shown in Figure A.2, the spectra collected from different points at and around the specific points indicated by the dotted circles in Fig. A.1 showed minimal differences. Furthermore as shown in Table A.1 the REIP and CIELAB values determined from these spectra are within acceptable limits of variation (20 plant samples tested). Hence, a slight deviation in position of the spectral collection points will not have any marked effect on

the result. Furthermore, the spectra need to be collected from the youngest fully expanded leaves which are normally 2.5 cm in width and 3.5 cm in length suggesting that the errors involved due to differences in collection point are minimal.

Figure A.1 Spectrum collection position on a leaf sample.

Figure A.2 Spectra collected from standard positions and their neighboring area.

Table A.1 REIP and CIELAB values of the spectra shown in Figure A.2.

REIP	L^*	a^*	b^*
710	30 ~ 31	-44 ~ -45	18 ~ 19

List of Publications

1. Bingqing Li, Oi Wah Liew, Anand Krishna Asundi (2006). Pre-visual detection of iron and phosphorus deficiency by transformed reflectance spectra, *Journal of Photochemistry and Photobiology B: Biology* vol.85: 131–139.
2. Jenny P. C. Chong, Oi Wah Liew, Bingqing Li & Anand Krishna Asundi (2005). Development of transgenic ‘Indicator Plants’ for fiber optic detection of drought stress conditions, *Biothailand 2005: Biotechnology, Challenges in the 21st Century*; 2–5 Nov 2005, Bangkok, Thailand.
3. Bingqing Li, Oi Wah Liew, Anand Krishna Asundi (2005). Early detection of calcium deficiency in plants using red edge position, *Proceedings of SPIE* vol.5996: 091–098.
4. Bingqing Li, Oi Wah Liew, Anand Krishna Asundi (2004). Use of reflectance spectroscopy for early detection of calcium deficiency in plants, *Proceedings of SPIE* vol.5852: 693–697.