<table>
<thead>
<tr>
<th>項目</th>
<th>内容</th>
</tr>
</thead>
<tbody>
<tr>
<td>タイトル</td>
<td>高等植物のクオリケンタス構造を介した遺伝子発現システムの開発とその応用に関する研究</td>
</tr>
<tr>
<td>著者</td>
<td>西城 隆憲</td>
</tr>
<tr>
<td>キーワード</td>
<td>高等植物, 遺伝子発現, クオリケンタス</td>
</tr>
<tr>
<td>インデックス</td>
<td>長崎大学, 博士, 学術</td>
</tr>
<tr>
<td>本番号</td>
<td>35207</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10069/35207">http://hdl.handle.net/10069/35207</a></td>
</tr>
</tbody>
</table>
Development of copper-inducible gene expression system for higher plants and its application study

March, 2015

Graduate School of Fisheries Science and Environmental Studies, Nagasaki University

Takanori SAIJO
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background and Objectives</td>
<td>3</td>
</tr>
<tr>
<td>Chapter 1 Development of an improved copper-inducible gene expression system</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>7</td>
</tr>
<tr>
<td>Results</td>
<td>12</td>
</tr>
<tr>
<td>Discussion</td>
<td>28</td>
</tr>
<tr>
<td>Chapter 2 Application study to control of flowering time</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>31</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>32</td>
</tr>
<tr>
<td>Results</td>
<td>33</td>
</tr>
<tr>
<td>Discussion</td>
<td>36</td>
</tr>
<tr>
<td>Chapter 3 Application study to a new detection tool for bioavailable copper in soil</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>37</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>39</td>
</tr>
<tr>
<td>Results</td>
<td>41</td>
</tr>
<tr>
<td>Discussion</td>
<td>49</td>
</tr>
<tr>
<td>Concluding Remarks</td>
<td>50</td>
</tr>
<tr>
<td>References</td>
<td>51</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>57</td>
</tr>
</tbody>
</table>
Background and Objectives

Copper is an essential element for all known living organisms, including humans and other animals. Though necessary at low concentrations, it is potentially toxic at higher levels (Dorsey et al. 2004). To develop a simple monitoring tool for copper content in environment, I focused on copper-inducible reporter gene expression system.

The potential use of the bacterium *Achromobacter* sp. AO22 as a copper biosensor has been previously reported (Ng et al. 2012). However, the assay with this microbe is far limited to a liquid suspension culture, and before its validation for environmental use, there are many challenging details to be investigated, including the stability of the strain, reproducibility of the assays, and ease of use. Meanwhile, a copper-inducible gene expression system that is based on regulation of the yeast metallothionein gene was first applied in tobacco by Mett et al. (1993). However, to date, no further successful application has been available, with the exception of their studies on controlled nodule asparagine concentration (Mett et al. 1996) and cytokinin production (McKenzie et al. 1998). Moreover, some researchers have reported that this copper-inducible system is not effective due to the low induction level of gene expression (Boetti et al. 1999, Granger et al. 2000 and 2001, Mohamed et al. 2001).

Considering a potential simplicity and low cost of monitoring method, I selected transgenic plant rather than microbe, and decided to start with improving the copper-inducible system reported by Mett et al. (1993 and 1996). *Arabidopsis* was selected as a target plant since this plant is easy to introduce foreign genes, can be grown in a small space with a short generation cycle, and its acceptable amount of copper is relatively high among plants. Essential points for improvement were to achieve high levels of reporter gene expression in a dose-dependent manner so that it can be detected easily, and to maintain the basal expression to negligible level in the absence of copper, for preventing false positive.

If the system is well improved, it can be useful not only as a monitoring tool, but also in various fields such as the efficient production of value-added proteins or metabolites in plant culture cells, physiological regulation of plant, analysis of gene function, and phytoremediation. To examine the potential application, I introduced an improved system into tobacco BY-2. Furthermore, I attempted to regulate floral transition by copper treatment since the control of flowering time is one of the most important technologies for improvement of crop yields and for the sustainable agronomic production (Jung and Muller 2009). Another purpose of this trial was to confirm how low the background expression level is in the absence of copper, since it requires tightly regulated gene
expression systems because floral transition is triggered by, for example, *FT*, which encodes a strong promoter of flowering, “florigen.”

After well characterizing, I evaluated the newly developed system for the potential use as a monitoring tool, by transferring to soil supplemented with various amounts of copper. Most regulations are based only on the physico-chemical data of samples extracted from soil by hydrochloric or nitric acid. Though physico-chemical methods of detection are highly sensitive and accurate, they have limited applicability in the biological context, since they cannot distinguish between the total amount in the environment and the actual amount available for biological use. Therefore, the development of simple and more sensitive bioassays for copper is needed to complement the existing physico-chemical techniques.

In this dissertation, I describe key points I found during development of an improved copper-inducible gene expression system, and demonstrate the potential application to regulate floral transition and to detect bioavailable copper in soil.
Chapter 1
Development of an improved copper-inducible gene expression system

Introduction

Effective gene expression systems are necessary for the analysis of gene functions and for agricultural applications. Constitutive promoters, such as the cauliflower mosaic virus (CaMV) 35S promoter, have been widely used for the expression of transgenes in plants (Odell et al. 1985). However, constitutive promoters are unsuitable for the expression of genes that are either highly deleterious or lethal when expressed inappropriately. To avoid such adverse effects, a number of regulated gene expression systems have been developed for plants (Boetti et al. 1999; Corrado et al. 2009; Moore et al. 2006; Padidam 2003). Ideal systems employ low cost, non-toxic inducers with fully controllable application. Such systems also include negligible basal expression of the target gene, but result in reversible dose-dependent expression that has the potential to achieve high levels of gene expression.

Earlier attempts to regulate transgene expression involved endogenous promoters that respond to hormones (Wyatt et al. 1993), light (Feinbaum et al. 1991), pathogens (Van de Rhee et al. 1990), temperature (Czarnecka et al. 1989), or drought (Yamaguchi-Shinozaki et al. 1993). Although these promoters are useful in some situations, such as the enhancement of stress tolerance, their inducers also have pleiotropic effects. Several laboratories have developed chemical-inducible systems that combine functional domains from non-plant sources. These systems, which include tetracycline-inducible (Gatz et al. 1992), dexamethasone-inducible (Aoyama et al. 1997), β-estradiol-inducible (Zuo et al. 2000), ethanol-inducible (Caddick et al. 1998), and ecdysteroid agonist-inducible systems (Tavva et al. 2006), are powerful tools for basic research in molecular biology and for biotechnological applications. However, all of these systems have some disadvantages (Moore et al. 2006; Padidam 2003; Tang et al. 2004; Zuo and Chua 2000). For example, the dexamethasone-inducible system has been reported to cause growth defects in tobacco (Amirsadeghi et al. 2007). Some inducers are expensive, whereas for others there are difficulties in application, with the inducer penetrating into or spreading on plants. In addition, most of these systems are induced by compounds that are not suitable for agricultural use, except for the ecdysteroid agonist-inducible systems. The inducer of these systems, methoxyfenozide, is registered as an insecticide. The ecdysteroid
agonist-inducible systems have been used in several different crop plants as well as in model crops (Semenyuk et al. 2010; Yang et al. 2012), including in activating expression of genes that control plant development and physiology (Tavva et al. 2007).

While methoxyfenozide is an artificial chemical, copper is one of the essential microelements as it occurs as part of the prosthetic groups of several enzymes. Although prolonged exposure of plants to relatively high copper concentrations causes a toxic response, copper is readily taken up by plants, easy to apply, inexpensive, and already registered as a fungicide for field use. Therefore, if non-toxic concentrations of copper can be used as an inducer of gene expression systems, this would constitute a genuinely powerful tool. Although a copper-inducible system was first applied in tobacco by Mett et al. (1993), to date, no further successful application has been available, with the exception of their studies on controlled nodule asparagine concentration (Mett et al. 1996) and cytokinin production (McKenzie et al. 1998). Moreover, some researchers have reported that this copper-inducible system is not effective for the following reasons: the target gene expression did not respond to copper in poplar (Mohamed et al. 2001); copper failed to induce GFP expression in BY2 cells (Granger et al. 2000); 100 μM copper resulted in an increase in GUS expression of only 1.2- to 4.6-fold in tobacco leaf protoplasts (Boetti et al. 1999); and GFP expression in Arabidopsis varied between plant lines, between different regions of a single plant, and between different tissues within a region (Granger et al. 2001).

To develop an effective copper-inducible system, I tested various copper-inducible systems expressing GFP as a reporter gene in transgenic Arabidopsis and identified some of the reasons why previously reported systems failed to work effectively in a reproducible manner in plants. Our newly developed copper-inducible system was investigated to determine its metal ion selectivity, dose-response, time course, effects of copper application timing, and potential for field use. Various applications of this system can be expected; however, I first attempted to demonstrate copper-inducible GFP accumulation in tobacco BY-2 cells because these cells are useful in basic research and are an important host for producing value-added proteins or metabolites.
Material and Methods

Plasmid construction

All primers and probes used in PCR experiments are listed in the Table 1-1.

Table 1-1  A list of all primers and probes used in PCR experiments.

<table>
<thead>
<tr>
<th>Primers for PCR</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE1-1F</td>
<td>5’-</td>
<td>ATGGATCCATGGTCGTAATTAACGGG</td>
<td>-3’</td>
</tr>
<tr>
<td>ACE1-1RC</td>
<td>5’-</td>
<td>TGGAGCCTCTTTATTTGTGAATGTGATTTATG</td>
<td>-3’</td>
</tr>
<tr>
<td>ACE1-2RC</td>
<td>5’-</td>
<td>AACTCGAAGTTGTAATGTGAGTTATGCG</td>
<td>-3’</td>
</tr>
<tr>
<td>VP16-1F</td>
<td>5’-</td>
<td>ACGGCTCCACCACGACGAGTC</td>
<td>-3’</td>
</tr>
<tr>
<td>VP16-1RC</td>
<td>5’-</td>
<td>CTACCCACCGTACTCGTCAATTCC</td>
<td>-3’</td>
</tr>
<tr>
<td>VP16-2F</td>
<td>5’-</td>
<td>GGACGAACTCCACTTAGACCGG</td>
<td>-3’</td>
</tr>
<tr>
<td>VP16-2RC</td>
<td>5’-</td>
<td>CCGTCTAAGTGGAGGTCTGCC</td>
<td>-3’</td>
</tr>
<tr>
<td>VP16-3F</td>
<td>5’-</td>
<td>TACCTCAGTCAACCGCTCACCACGACGATGGT</td>
<td>-3’</td>
</tr>
<tr>
<td>VP16-3RC</td>
<td>5’-</td>
<td>AAGAGCCTTCTTACCACCGTACTCGTCAATTCCAAAG</td>
<td>-3’</td>
</tr>
<tr>
<td>46bp-1F</td>
<td>5’-</td>
<td>TAGTATACGCAAGACCTCTCTCTATATTAAGG</td>
<td>-3’</td>
</tr>
<tr>
<td>46bp-1RC</td>
<td>5’-</td>
<td>ATCCTCTAGAGTCCCCCGTGTC</td>
<td>-3’</td>
</tr>
<tr>
<td>90m-1F</td>
<td>5’-</td>
<td>GCTATGACCATGATTACGGAAGCTTG</td>
<td>-3’</td>
</tr>
<tr>
<td>90m-1RC</td>
<td>5’-</td>
<td>CATTGTTATATCTCTTGGATCCCGTG</td>
<td>-3’</td>
</tr>
<tr>
<td>90m-2F</td>
<td>5’-</td>
<td>TAGATATCTCCACGTTCCATAAGGGAC</td>
<td>-3’</td>
</tr>
<tr>
<td>90m-2RC</td>
<td>5’-</td>
<td>AATCTAGCTAGCTGCGACGTC</td>
<td>-3’</td>
</tr>
<tr>
<td>71bp-1F</td>
<td>5’-</td>
<td>TGCTCTAGAGTATTTTTACAAAATATACCACCAACCACAC</td>
<td>-3’</td>
</tr>
<tr>
<td>71bp-1RC</td>
<td>5’-</td>
<td>AAGGATCCTCGTATGGTAGGAATGTATCAAATT</td>
<td>-3’</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primers and probes for real-time PCR</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>sGFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S01F</td>
<td>5’-</td>
<td>TCCGCCCTGAGCGAAAGAC</td>
<td>-3’</td>
</tr>
<tr>
<td>S01</td>
<td>5’-</td>
<td>FAM-CCAACGAGAGACGAGG-MGB</td>
<td>-3’</td>
</tr>
<tr>
<td>S01RC</td>
<td>5’-</td>
<td>GAACTCGAGACGAGACGATGTG</td>
<td>-3’</td>
</tr>
<tr>
<td>AtACT2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S03F</td>
<td>5’-</td>
<td>CCGTGGTCCATTCTTGCTT</td>
<td>-3’</td>
</tr>
<tr>
<td>S03</td>
<td>5’-</td>
<td>VIC-CCTAGCACATACAC-MGB</td>
<td>-3’</td>
</tr>
<tr>
<td>S03RC</td>
<td>5’-</td>
<td>CGGCCCTTGGGAGACGACCAT</td>
<td>-3’</td>
</tr>
<tr>
<td>FT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TaqMan gene expression assay of FT (Assay ID; At02224075_g1)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Construction of transcription factor gene expression cassettes

ACE1 was replaced with GUS from pBI221 to create p35S-ACE1-NOS. The NOS terminator was replaced with a CR16 terminator (Yamamoto et al. 1997) to create p35S-ACE1-CR. VP16AD was inserted downstream of ACE1 to create p35S-ACE1/VP16AD-CR.
**Construction of reporter GFP gene expression cassettes**

sGFP was cut out of plasmid CaMV35S-sGFP(S65T)-NOS3', kindly provided by Dr. Y. Niwa of Shizuoka Prefecture University, and was replaced with the GUS of pBI221 to create p35S-sGFP. The -830-bp to -90-bp region of the 35S promoter was replaced with synthetic MRE (metal-responsive element) oligonucleotides (MRE-1F, 5’-agcttagctgctttttctcggaacgctgcaaaagactagat-3’ and MRE-1RC, 5’-atctagtcttttttgtggaacgctgcaaaagactagat-3’) to create pMRE/35S(-90)-sGFP. The blunt-ended MRE oligonucleotides were inserted into EcoRV-digested pMRE/35S(-90)-sGFP to arrange MRE sequences twice. The twice-repeated MRE sequences were cut out with HindIII and EcoRV, blunted, and inserted into the EcoRV site to create pMRE4/35S(-90)-sGFP, in which the MRE sequences were repeated four times in a forward direction. The 35S(-46) minimal promoter was replaced with the 35S(-90) minimal promoter of pMRE4/35S(-90)-sGFP to create pMRE4/35S(-46)-sGFP. The 35S(-90m) minimal promoter was replaced with the 35S(-90) minimal promoter of pMRE4/35S(-90)-sGFP to create pMRE4/35S(-90m)-sGFP. The 35S(-90m) minimal promoter has mutations at the ASF1 binding site of the 35S(-90) minimal promoter (Gardner et al. 2009; Lam et al. 1989). The To71 sequence was amplified from piLerG3 (Tamai et al. 2001), kindly provided by Dr. M. Mori of Ishikawa Prefecture University, and was inserted into XbaI/BamHI-digested pMRE4/35S(-46)-sGFP and pMRE4/35S(-90m)-sGFP to create pMRE4/35S(-46)-To71sGFP and pMRE4/35S(-90m)-To71sGFP.

**Construction of binary vectors**

Transcription factor gene expression cassettes were cut out of p35S-ACE1-CR and p35S-ACE1/VP16AD-CR using HindIII and EcoRI restriction enzymes. After pMRE/35S(-90)-sGFP, pMRE4/35S(-46)-sGFP, pMRE4/35S(-46)-To71sGFP, pMRE4/35S(-90m)-To71sGFP, and pMRE4/35S(-90m)-To71FT were digested with HindIII and blunted, synthetic KXS oligonucleotides (KXS-1F, 5’-ggtacctcagctgac-3’ and KXS-1RC, 5’-gctgacagtcttacctac-3’) were inserted into them to create pKXS-MRE/35S(-90)-sGFP, pKXS-MRE4/35S(-46)-sGFP, pKXS-MRE4/35S(-46)-To71sGFP, and pKXS-MRE4/35S(-90m)-To71sGFP. The sGFP gene expression cassettes were cut out of these plasmids using KpnI and EcoRI restriction enzymes. The GUS expression cassette contained in pBI121 (Clontech, Mountain View, CA, USA) was replaced with synthetic HEK oligonucleotides (HEK-1, 5’-agcttgcatcgacgtgctacagcagatagacgctgac-3’ and HEK-1RC, 5’-aatgctgcatcagcagcatcagcaattc-3’) to create pBI121-HEK. The transcription factor gene expression cassettes and the sGFP gene expression cassettes were inserted
into the HindIII/KpnI-digested pBI121-HEK to obtain vectors in which the transcription factor gene expression cassettes and the sGFP gene expression cassettes were ligated in a tail-to-tail orientation (Fig. 1-1). The vector derived from p35S-ACE1-CR and pKXS-MRE/35S(-90)-sGFP was designated as pSUM21-sGFP. The vector derived from p35S-ACE1-CR and pKXS-MRE4/35S(-46)-sGFP was designated as pSUM24-sGFP. The vector derived from p35S-ACE1/VP16AD-CR and pKXS-MRE/35S(-46)-sGFP was designated as pSUM44-sGFP. The vector derived from p35S-ACE1/VP16AD-CR and pKXS-MRE4/35S(-46)/To71sGFP was designated as pSUM48-sGFP. The vector derived from p35S-ACE1/VP16AD-CR and pKXS-MRE/35S(-90m)-To71sGFP was designated as pSUM46-sGFP.

**Plant Transformation and growth conditions**

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used in this study. Unless otherwise indicated, all seeds were sterilized with diluted bleach, rinsed with sterile water, maintained at 4°C for 3 days in the dark, and sown on 0.8% agar plates containing MS salts, vitamin B₅, 2.5 mM MES, and 2% sucrose. Plates were incubated in a growth room at 23°C under a 23/1-h photoperiod at 65 μmol photons m⁻² sec⁻¹. The appropriate vector constructs were introduced into *Agrobacterium tumefaciens* C58C1 and then transformed into *Arabidopsis* by vacuum infiltration. Seeds were sown on selective plates containing 25 mg/l kanamycin. The T₂ generation was analyzed by segregation analysis to determine the number of T-DNA copies in each independent transformant. Transformants that resulted in segregation of the kanamycin-resistant marker, consistent with the expected Mendelian ratio of 3:1 for a single integration event, were selected for further analysis. The seeds from individual plants were collected separately. From these, each line was tested for kanamycin resistance and non-segregating T₃ progeny were selected for further characterization.

Tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) suspensions were diluted 95-fold with a modified Linsmaier and Skoog medium (LSD medium) that was supplemented with 2,4-D at weekly intervals (Takeda et al. 1992). The cell suspensions were agitated on a rotary shaker at 130 rpm at 23°C in the dark. A suspension of 5-day-old BY-2 cells was filtrated onto filter paper, and the cells bombarded with gold particles (1.0 μM) that were coated with the appropriate vector constructs, using a particle-delivery system (PDS-1000/He; Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. Filtered BY-2 cells were placed 6 cm below the stopping screen and bombarded in a vacuum of 28 inches Hg at a helium pressure of 1100 psi. Following bombardment, cells were diluted in LSD medium and kept in the dark at 23°C.
for 3 days. The cells were subsequently resuspended in 10 ml LSD medium, and then plated onto solid LSD medium containing 30 mg/l kanamycin. Calluses, which appeared after 20 days, were transferred to new plates and cultured independently until they reached approximately 1 cm in diameter. Half of the calluses were then transferred to solid LSD medium containing 100 μM CuSO₄. After 3 days of incubation, a cell line suitable for further examination was selected by observing GFP fluorescence under fluorescence microscopy.

**Copper sulfate treatments**

Unless otherwise indicated, transgenic plants were transferred to agar medium that was or was not supplemented with 100 μM CuSO₄. For the metal ion selectivity study, transgenic plants were sown on agar plates containing “metal-free medium” that consisted of 20 mM NH₄NO₃, 19 mM KNO₃, 1.25 mM KH₂PO₄, 100 μM H₃BO₃, 1 μM Na₂MoO₄, 2.5 mM MES, and 2% sucrose. Twelve-day-old seedlings were transferred to the same agar medium supplemented with 20 μM Cd²⁺ or Pb²⁺; 100 μM Co²⁺, Cu²⁺, Ag⁺, or Zn²⁺; or 500 μM Mn²⁺. In the foliar spraying test, 0.5 ml of 100 μM CuSO₄ solution was used per plant. In the soil drenching test, 2.0 ml of 100 μM CuSO₄ solution was used per plant.

**Microscopy**

Transgenic BY-2 cells and 10-day-old seedlings of T₃ Arabidopsis plants were transferred to agar medium that was or was not supplemented with 100 μM CuSO₄. Fluorescent and bright-field images were observed after 3–5 days of incubation using fluorescence microscopy (MVX10: Olympus, Tokyo, Japan, or VB-G05: Keyence, Osaka, Japan) and photographed using a ccd camera (DP71: Olympus, Tokyo, Japan, or VB-7010: Keyence, Osaka, Japan). The exposure time for fluorescent images was 1 sec.

**Real-time PCR analysis**

Total RNA was isolated from seedlings (including both roots and above-ground portions) using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions. The seedling samples were collected into 2 mL Eppendorf tubes from agar medium or soil, immediately frozen in liquid nitrogen, and then stored in deep freezer until extraction. One microgram of total RNA was converted to cDNA using
the ReverTra Ace (TOYOBO, Osaka, Japan) primed with oligo-dT, following the manufacturer’s instructions. MGB-TaqMan probes and primers (Table 1.1) were designed based on cDNA sequences from Arabidopsis using Primer Express 3.0 software (Applied Biosystems, Carlsbad, CA, USA). Specific amounts of mRNA were quantified by TaqMan real-time PCR (7500 Fast Real-time PCR system) using AtACT2 (GenBank Accession NM180280) for normalization. FAM and VIC reporter (5’ end) dyes were used for the MGB-TaqMan probes of sGFP and AtACT2, respectively. The relative amounts of mRNA were calculated using the comparative C_T method.

**Immunoblotting analysis**

Ground BY-2 callus was incubated at 99°C in sample buffer [65 mM Tris-HCl, pH 6.75, 4% (w/v) SDS, 10% (v/v) b-mercaptoethanol, 20% (v/v) glycerol] for 3 min. After centrifugation at 15,000 × g for 10 min, the supernatant was subjected to SDS-PAGE using 15%/25% polyacrylamide gels, transferred to a PVDF membrane, and analyzed by immunoblotting with anti-GFP peptide antibody, PA1-980A (Affinity BioReagents, Rockford, IL, USA), and horseradish peroxidase-conjugated secondary antibody, NA934VS (GE Healthcare, Buckinghamshire, UK). A chemiluminescence image was obtained using a ChemiDoc XRS (BIO-RAD, Hercules, CA, USA)
Results

Development of an efficient copper-inducible gene expression system

The copper-inducible gene expression system described here essentially consists of two elements: (1) the yeast activating copper-metallothionein expression (ACE1) gene, which encodes the copper-regulated transcription factor that is under the control of a constitutive promoter, and (2) a gene of interest that is under the control of a chimeric promoter. The chimeric promoter consists of a minimal promoter linked to a metal responsive element (MRE) that contains ACE1 binding sites. To develop an efficient gene expression system, I decided to investigate the effects of the following components: the number of MRE repeats, a minimal promoter sequence, the addition of VP16AD to ACE1, and the insertion of the To71 sequence upstream of the gene of interest. For the above purpose, five binary vectors were constructed (Fig. 1·1). The T-DNA of each vector was introduced into Arabidopsis via vacuum infiltration, and between 15 and 28 independent kanamycin-resistant transformants were obtained. Of these primary transformants, 7–14 transformants were selected by kanamycin-resistant segregation in the T2 generation. Ten-day-old seedlings of T2 plants, which were expected to carry single-copy T-DNA, were transferred to agar medium that was or was not supplemented with 100 μM CuSO4. Six hours after transfer, the sGFP transcripts in copper-treated seedlings of lines 21, 24, 44, 48, and 46 increased by 5, 9, 273, 627, and 325-fold, respectively, when compared with non-treated controls (Fig. 1·2).
Fig. 1-1  Schematic representation of copper-inducible gene expression system vectors.

The sGFP gene was inserted downstream of a target promoter that consisted of either 1 or 4 copies of MRE fused to 35S(-90), 35S(-46), or 35S(-90m) minimal promoter. The To71 sequence was inserted between the minimal promoter and the sGFP gene. The amino acid sequence of the chimeric transcriptional activator consisted of the full length of ACE1 and the VP16 activation domain of herpes simplex virus. pSUM21-sGFP, pSUM24-sGFP, pSUM44-sGFP, pSUM48-sGFP, and pSUM46-sGFP are represented by 21, 24, 44, 48, and 46, respectively. The symbol “*” indicates a mutation region of the ASF1 binding site in the 35S promoter.

35S pro, the CaMV 35S promoter;
ACE1, the transcription factor for the activating copper-metallothionein expression;
CDBD, the copper and DNA binding domain;
AD, the activation domain;
MRE, the metal responsive element;
To71, the 5'UTR sequence of RNA-dependant RNA polymerase gene of tomato mosaic virus;
CR16 ter, the carrot CR16 terminator;
NOS ter, the NOS terminator;
Kanr, the kanamycin resistance gene.
Six 10-day-old seedlings of T2 plants were transferred to agar medium that was (gray columns) or was not (white columns) supplemented with 100 μM CuSO4. Total RNA was extracted after 6 hours of incubation, and was analyzed by real-time PCR. Transgenic plants that were transformed with pSUM21-sGFP, pSUM24-sGFP, pSUM44-sGFP, pSUM48-sGFP, and pSUM46-sGFP are represented by 21, 24, 44, 48, and 46, respectively. The amount of sGFP mRNA was normalized to that of actin. The bars indicate the SD of from 7–14 independent lines. Fold induction ratios shown on the top of the bars were calculated by dividing amount of sGFP mRNA in the presence of copper by that in the absence of copper.
The line 21 plants carrying the ACE1 gene and the sGFP gene driven by the 35S(-90) minimal promoter linked to a single MRE showed only weak fluorescence in the roots after treatment with copper for 3 days (Fig. 1-3, 1-8). To enhance the copper-induced expression of GFP, MRE sequences were arranged four times. To reduce the basal expression level of GFP, the ASF1 binding site was removed from the 35S(-90) minimal promoter. Although these modifications resulted in some improvement, the induction ratio of line 24 was still low (Fig.1-2). When VP16AD was fused to ACE1, as in lines 44, 48, and 46, the induction ratios were significantly improved. Moreover, to enhance translational efficiency, the Tb71 sequence, the 5′-untranslated region of the 130k/180k gene of tomato mosaic virus, was inserted between the minimal promoter and the sGFP gene. As expected, the number of plant that shows good response to the copper was higher in line 48 than line 44 (Fig. 1-9). Moreover, GFP fluorescence of the line 48 plant was stronger than that of the line 44 plant in the presence of copper. Surprisingly, in addition to the improvement of translational efficiency, the basal expression levels of both lines 48 and 46 were lower than that of line 44 (Fig. 1-2). After treatment with copper for 3 days, line 48 plants showed strong GFP fluorescence in whole plants, while no GFP fluorescence was detected in the absence of copper (Fig. 1-3, 1-9). Some plants of line 48 and 46 in the presence of copper showed stronger GFP fluorescence than any plants carrying the GFP gene driven by 35S promoter (Fig. 1-9, 10, 11). At first, assuming that the length of minimal promoter might affect the induction efficiency, ASF1 cite was removed by the two different ways. (1) 35S(-46) as in line 24, 44 and 48, the deletion of sequence from -90 bp to -47 bp. (2) 35S(-90m) as in line 46, the introduction of mutations to the ASF1 binding cite as indicated in the Fig.1·1. However, it turned out there was no significant difference in the induction level between line 48 and line 46 (Fig. 1-9, 10). Therefore, one representative line, line 46-6, was selected for further analyses.
Fig. 1-3  Induced GFP expression in *Arabidopsis thaliana*.

Ten-day-old seedlings of T$_3$ plants were transferred to agar medium that was (b, d, f, h) or was not (a, c, e, g) supplemented with 100 µM CuSO$_4$. Bright-field (a-d) and fluorescent (e-h) images were obtained after 3 days of incubation. Transgenic plants in (a, b, e, f) and (c, d, g, h) were transformed with *pSUM21-sGFP*, and *pSUM48-sGFP*, respectively.
Characterization of the new system in Arabidopsis

First, I studied metal ion selectivity to examine whether the fusion of VP16AD to ACE1 affects the selectivity. To test the response of sGFP transcription to various metal ions, T₃ seeds of line 46-6b were germinated on metal-free agar medium. Twelve-day-old seedlings were transferred to agar medium supplemented with various metal ions (20 μM Cd²⁺ or Pb²⁺; 100 μM Co²⁺, Cu²⁺, Ag⁺ or Zn²⁺; or 500 μM Mn²⁺). The natural ACE1 has been shown to respond to Cu²⁺ and Ag⁺ (Furst et al. 1988). Six hours after transfer, the sGFP transcripts in copper-treated seedlings increased by 224-fold, when compared with non-treated controls (Fig. 1-4). The Ag⁺ treatment resulted in a 38-fold induction. The other metal ion treatments resulted in almost no induction, indicating that our newly developed copper-inducible system had high selectivity for metal ions.

![Fig. 1-4 Selective response to heavy metal ions.](image)

T₃ seeds of line 46-6b were germinated on metal-free agar medium. Twelve-day-old seedlings were transferred to the agar medium supplemented with 20 μM Cd²⁺ or Pb²⁺; 100 μM Co²⁺, Cu²⁺, Ag⁺, or Zn²⁺; or 500 μM Mn²⁺. Total RNA was extracted after 6 hours of incubation, and analyzed using real-time PCR. The amount of sGFP mRNA was normalized to that of actin.
Next, I studied the dose response of this system. Ten-day-old seedlings of line 46-6b were transferred to agar medium supplemented with various concentrations of CuSO₄ (0.1, 1, 2, 5, 10, 20, 50, 100, 200, 500, or 1000 μM) and incubated for 6 hours. As shown in Fig. 1-5a, the sGFP transcripts in copper-treated seedlings increased with increasing copper concentrations from 1 μM to 500 μM. The dynamic range of copper concentration therefore spans two orders of magnitude and should allow for quantitative control over induced expression. A 100 μM copper treatment was sufficient to induce the highest level of expression, while 50% of the maximum expression was obtained with 20 μM copper. Induction appeared to saturate at 500 μM of copper, and higher concentrations did not significantly increase expression levels. Additionally, at copper concentrations above 500 μM, the seedlings exhibited signs of stress that were probably due to copper toxicity (stunted growth, chlorosis, and shortened roots). Copper toxicity was related to exposure time and copper concentration. I detected only weak copper toxicity after treatment with 100 μM copper for 3 days, as shown in Fig. 1-3. In subsequent experiments, 100 μM copper was used to induce expression.

To investigate induction kinetics, 10-day-old seedlings of line 46-6b were then transferred to agar medium supplemented with 100 μM CuSO₄ and incubated for various periods (0.1, 1, 2, 4, 6, 12, 24, 48, or 96 hours). As shown in Fig. 1-5b, sGFP transcripts appeared within an hour, reached the maximum level at 12 hours, and remained relatively constant thereafter. To evaluate the time course of down-regulation, seedlings treated with 6 hours of induction were transferred to an agar medium that did not contain CuSO₄ and were incubated for various periods (6, 18, 42 and 90 hours). The sGFP transcripts were significantly lower 6 hours after transfer than they were immediately after induction, gradually declined in a time-dependant manner, and had reached basal levels by 90 hours after transfer (Fig. 1-5 (b)).
Fig. 1-5  Dose-dependent and time-course of copper induction.

(a) Ten-day-old seedlings of line 46-6b were transferred to agar medium supplemented with 0.1, 1, 2, 5, 10, 20, 50, 100, 200, 500, or 1000 μM CuSO₄. Total RNA was extracted after 6 hours of incubation, and was analyzed using real-time PCR.

(b) Ten-day-old seedlings of line 46-6b were transferred to agar medium supplemented with 100 μM CuSO₄. Total RNA was extracted after 0.1, 1, 2, 4, 6, 12, 24, 48, or 96 hours of incubation, and was analyzed using real-time PCR (circles). After 6 hours of incubation with 100 μM CuSO₄, half of the seedlings were transferred to agar medium that did not contain CuSO₄. Total RNA was extracted after 6, 18, 42, and 90 hours of incubation, and was analyzed by real-time PCR (triangles). The amount of sGFP mRNA was normalized to that of actin. The bars indicate the SD of 2 measurements.
In addition, to investigate the effects of the timing of copper application, T₃ seeds of line 46·6b were germinated on agar medium supplemented with 0, 50, or 100 μM CuSO₄. Total RNA was extracted for real-time PCR analysis 7 days after sowing. In addition, 7-, 14-, or 21-day-old seedlings were transferred to agar medium supplemented with or without 100 μM CuSO₄ and incubated for 6 hours. As shown in Fig. 1·6 (a), continuous copper induction with 50 μM or 100 μM CuSO₄ in germination medium resulted in a 649- or 1140-fold increase in sGFP transcripts, respectively. The sGFP transcription levels were dependent on the copper concentration of the germination medium. Pulse copper induction resulted in 1407-, 695-, and 286-fold increases in sGFP transcripts in 7-, 14-, and 21-day-old seedlings, respectively, when compared with sGFP transcripts in non-treated 7-day-old seedlings (Fig. 1·6 (a)). sGFP transcription levels decreased as seedling age at induction increased.

Finally, to examine the potential of copper induction under field conditions, 21-day-old seedlings of line 46·6b were transferred to soil and supplemented with a 1/1000 dilution of Hyponex. Almost no induction of sGFP transcripts was detected in the seedlings 6 hours after transfer (Fig. 1·6 (a)). In addition, 18-day-old seedlings were transferred to soil and treated with 100 μM CuSO₄ by foliar spraying or by soil drenching. The sGFP transcripts increased by 703- and 414-fold 6 hours after foliar spraying and soil drenching, respectively, when compared with sGFP transcripts in non-treated 18-day-old seedlings (Fig. 1·6 (b)).
(a) T<sub>3</sub> seeds of line 46-6b were germinated on agar medium that was or was not supplemented with 50 or 100 µM CuSO<sub>4</sub>. Total RNA was extracted 7 days after sowing. In addition, 7-, 14-, and 21-day-old seedlings of line 46-6b were transferred to agar medium that was or was not supplemented with 100 µM CuSO<sub>4</sub>, or to soil (*, †). Seedlings transferred to soil were supplemented with a 1/1000 dilution of Hyponex (†). Total RNA was extracted after 6 hours of incubation, and was analyzed by real-time PCR.

(b) Eighteen-day-old seedlings of line 46-6b were transferred to soil and treated with 100 µM CuSO<sub>4</sub> by foliar spraying or by soil drenching. Six hours after treatment, total RNA was extracted and analyzed using real-time PCR. The amount of sGFP mRNA was normalized to that of actin. The bars indicate the SD of 2 measurements.
Induced GFP expression in tobacco BY-2 cells

To examine the potential of the copper-inducible system for the production of value-added proteins or metabolites in plant culture cells, pSUM46-sGFP was introduced into tobacco BY-2 cells by particle bombardment. A number of kanamycin-resistant lines were obtained. A transgenic line that strongly expressed GFP in the presence of copper was transferred to a solid LSD medium with or without 100 μM CuSO₄. As shown in Fig. 1-7, strong GFP fluorescence was detected within 5 days of copper treatment. The induced level of GFP fluorescence was equal to or higher than that of a constitutively expressing line that contains 35S-sGFP (selected from an equivalent sized transgenic population). In contrast, GFP fluorescence was not detected in the absence of copper. Immunoblotting analysis confirmed induced accumulation of GFP in the pSUM46-sGFP line that was correlated with GFP fluorescence.

<table>
<thead>
<tr>
<th>SUM46-sGFP</th>
<th>35S-sGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

![GFP expression images](image)

**Fig. 1-7  Induced GFP expression in tobacco BY-2 cells.**

Transgenic cells were transferred to agar medium that was (+) or was not (−) supplemented with 100 μM CuSO₄. Fluorescent images (upper) were obtained after 5 days of incubation. Total proteins (10 μg) were extracted from the cells, and analyzed using immunoblotting with anti-GFP antibody and alkaline phosphatase-conjugated secondary antibody (lower). SUM46-sGFP and 35S-sGFP transgenic cells were transformed with pSUM46-sGFP and pBI-35S-sGFP, respectively.
Fig. 1-8  Induced GFP expression in line 21, 24, and 91
Fig. 1-9  Induced GFP expression in line 44, 48, and 91
Fig. 1-10  Induced GFP expression in line 46 and 91
Fig. 1-11  Induced GFP expression in line 91
GFP fluorescent (upper) and bright-field (lower) images of T₃ seedlings with or without 3 days of treatment with 100 μM copper. Transgenic plants that were transformed with pSUM21-sGFP, pSUM24-sGFP, pSUM44-sGFP, pSUM48-sGFP, pSUM46-sGFP, and pBI-35S-sGFP are represented by the numbers 21, 24, 44, 48, 46, and 91, respectively. For each construct, 6–9 independent homozygous lines are displayed.
Discussion

By using our newly developed copper-inducible gene expression system, the transcription of a target gene was strongly induced in the presence of copper, while the basal expression in the absence of copper was practically negligible; therefore, the system could be applied to regulate floral transition. The system increased induction by over 1,000-fold and provided quantitative control of target gene expression in a copper-specific manner. These data clearly demonstrate that our new system can be tightly regulated and highly responsive in plants. In transgenic tobacco BY-2 cells, GFP was highly accumulated in the presence of copper, whereas almost no GFP was detected in the absence of copper. A previous study showed that copper failed to induce GFP expression in BY2 cells (Granger et al. 2000). However, this is not surprising, as copper treatment resulted in a very low induction of GFP expression in transgenic Arabidopsis that carried the ACE1 gene and the sGFP gene driven by a 35S(-90) minimal promoter linked to a single MRE. This induction ratio is similar to that found in a previous study of tobacco leaf protoplasts exposed to 100 μM copper (Boetti et al. 1999). The variable copper-inducible expression of GFP in Arabidopsis (Granger et al. 2000) may also be due to this low induction ratio. Variation in transgene expression was observed even when using constitutive promoters, such as the CaMV 35S promoter (Moore et al. 2006; Fig. 1-11). This is probably due to a positional effect and/or a silencing. The range of variation might be wider in transgenic plants using the copper-inducible system than in plants using constitutive promoters; however, it does not differ from that of other inducible systems. For example, 5 elite lines were selected from 22 lines of transgenic Arabidopsis that were transformed with β-estradiol-inducible GFP expression vector (Zuo et al. 2000). This rate is similar to that obtained using our newly developed copper-inducible system. In our study, at least 5 of 14 transformants carrying the copper-inducible GFP expression vector (pSUM48-sGFP) exhibited strong GFP fluorescence throughout the plant when treated with copper, although only 1 of 7 transformants carrying the earlier type of copper-inducible GFP expression vector (pSUM21-sGFP) exhibited a relatively strong GFP fluorescence in the roots when treated with copper (Fig. 1-8, 9, 10, 11).

Modification of the copper-inducible system to include four repeated MREs and a shortened minimal promoter 35S(-46) resulted in an improvement in the induction ratio from 50-fold to 150-fold (Mett et al. 1996). Our follow-up experiment with Arabidopsis showed an improvement with a similar ratio. However, the induction ratio was still low. Although this modified system has been used to regulate the expression of the isopentenyl transferase gene in tobacco plants (McKenzie et al. 1998), there have not been any follow-up experiments using this modified system, and it is unclear whether it can be applied to transgene expression in other plants. It is probable that this modified
system could not be used to regulate the isopentenyl transferase gene in *Arabidopsis*. The copper-inducible system works well in yeast and mammalian cells without the addition of an activation domain such as VP16AD (Culotta et al. 1989; Labbe et al. 1999). Moreover, in yeast, the substitution of the activation domain of ACE1 with that of VP16 caused a reduction in the copper-inducible expression of a target gene (Pena et al. 1998). However, in plants, the addition of VP16AD to ACE1 was critical to the function of the copper-inducible system and the induction ratio was not significantly elevated until VP16AD was fused to ACE1. I suppose the addition of VP16AD was the most effective change compared with the previous studies. Moreover, the addition of VP16AD to ACE1 did not affect the metal ion selectivity, because this chimeric transcription factor responds only to copper and silver, as does natural ACE1 (Furst et al. 1988).

Interestingly, introduction of the *To71* sequence, which is similar to the TMV Ω sequence, into the 5′-untranslated region resulted in the reduction of the basal expression of the target gene. It has been reported that chloramphenicol acetyltransferase mRNA with the TMV Ω sequence exhibited greater sensitivity to micrococcal nuclease than that without the TMV Ω sequence (Gallie et al. 1988). Although no direct evidence has been obtained, the *To71* sequence might perhaps work as a potent mRNA destabilizer especially in the absence of copper while it worked as a translational enhancer.

The expression level of the reporter gene depended on the timing of copper application. The amount of *sGFP* transcripts in seedlings decreased as seedling age at induction increased. This suggests that more time is required to deliver copper throughout the plants when copper is applied via agar medium. The expression level of the reporter gene after foliar spraying of copper was higher than after soil drenching, indicating that copper is incorporated into plants through aerial parts. A previous study also reported that a lower concentration of copper is sufficient to induce the expression of a target gene when the copper is applied by foliar spraying than when it is applied via a nutrient solution (Mett et al. 1993). Copper as an inducer is superior to other inducers in ease of application, penetration and transportation, and cost. Moreover, copper is already used in the open field as a fungicide in concentrations ranging from 0.5 mM to 32 mM. In addition, almost no induction of *sGFP* transcripts was detected in seedlings that were transferred to soil and supplemented with an ordinary liquid nutrient. When the β-estradiol-inducible system was utilized for a target gene expression in rice, reduced induction was observed in leaves while good induction was observed in roots (Okuzaki et al. 2011). Although further evaluation in other plants, for example in rice, and investigations using practical target genes are required, transgenic plants carrying the new copper-inducible system have the potential to be used to control plant physiology, growth, flowering, flower color, germination, hormone response, stress tolerance, etc., in
the field. In addition, this system could be useful for real-time imaging of copper trafficking in plants. I am attempting to create transgenic rice that carries our newly developed copper-inducible system and are further developing systems that utilize the Gateway System (Invitrogen, Carlsbad, CA, USA) for the efficient analysis of gene function. I envisage its use will contribute significantly to both functional genomics and agribiotechnology.

The reporter gene expression responded to copper concentrations between 1 µM and 500 µM in a dose-dependent manner, and did not respond to 0.1 µM copper in Murashige and Skoog (MS) salts when using our newly developed copper-inducible system. A previous study reported that copper toxicity appeared after exposures of 3 and 20 days to 50 µM and 20 µM copper, respectively (Granger et al. 2001). In our dose-response study, the mRNA was assayed 6 hours after the addition of copper, before the appearance of copper toxicity. Additionally, I detected only weak copper toxicity in plants treated with 100 µM copper for 3 days, as shown in Fig. 1-3. The inducers of some inducible systems have been reported to cause toxicity in some cases (Amirsadeghi et al. 2007; Andersen et al. 2003; Kang et al. 1999; Roslan et al. 2001). Whereas any inducer is likely to influence the plant to some degree, inducers differ as to whether they create an obvious side effect. The acceptable degree of toxicity depends on the goal of induction. If inducers are used for the production of value-added proteins or metabolites in plants, toxicity is acceptable, as long as maximum production is achieved. In fact, transgenic tobacco BY-2 cells carrying pSUM46-sGFP that were treated with 100 µM copper for 5 days accumulated GFP at equal to or higher levels than 35S-GFP cells.
Chapter 2

Application study to control of flowering time

Introduction

I subsequently attempted to regulate floral transition by copper treatment. The control of flowering time is one of the most important technologies for improvement of crop yields and for the sustainable agronomic production (Jung and Muller 2009). It requires tightly regulated gene expression systems because floral transition is triggered by, for example, *FT*, which encodes a strong promoter of flowering, “florigen” (Corbesier et al. 2007; Jaeger and Wigge 2007; Lin et al. 2007; Mathieu et al. 2007; Tamaki et al. 2007; Notaguchi et al. 2008). In *Arabidopsis*, the *FT* gene is expressed in cotyledons and leaves in response to inductive long days. FT protein, together with a basic region/leucine zipper (bZIP) transcription factor FD, acts in the shoot apex to induce floral transition (Kobayashi et al. 1999; Kardailsky et al. 1999; Abe et al. 2005; Wigge et al. 2005). To date, there have been only a few reports on chemically tight control of floral transition. For example, application of the steroid dexamethasone (DEX) induced transgenic *Arabidopsis* floral transition via GIGANTEA protein fused to the ligand-binding domain of the rat glucocorticoid receptor (Gunl et al. 2009). On the other hand, an ethanol-inducible system was utilized for the inducible expression of *FTa1*, an *FT* orthologue from *Medicago truncatula* cv. Jester (Yeoh et al. 2011). However, DEX is obviously not suitable for field use, and in the latter case, care must be taken to avoid exposing the tissues or plants to ethanol vapours, otherwise induction is triggered inadvertently and inappropriately (Padidam 2003). In this report, I describe the successful application of a copper-inducible system, which enables the conditional production in plant cells and the control of plant morphology.
Material and Methods

**Plasmid construction**

All primers and probes used in PCR experiments are listed in the Table 1·1. FT was replaced with sGFP from pMRE4/35S(-90m)-To71sGFP to create pMRE4/35S(-90m)-To71FT. Transcription factor gene expression cassettes were cut out of p35S-ACE1/VP16AD-CR using HindIII and EcoRI restriction enzymes. After pMRE4/35S(-90m)-To71FT were digested with HindIII and blunted, synthetic KXS oligonucleotides (KXS-1F, 5′-ggtacctcgagtcgac-3′ and KXS-1RC, 5′-gctgactgagttacc-3′) were inserted into them to create pKXS-MRE/35S(-90m)-To71FT. The FT gene expression cassettes were cut out of these plasmids using KpnI and EcoRI restriction enzymes. The transcription factor gene expression cassettes and the FT gene expression cassettes were inserted into the HindIII/KpnI-digested pBI121-HEK to obtain vectors in which the transcription factor gene expression cassettes and the FT gene expression cassettes were ligated in a tail-to-tail orientation (Fig. 1·1). The vector derived from p35S-ACE1/VP16AD-CR and pKXS-MRE/35S(-90m)-To71FT was designated as pSUM46-FT.

**Plant Transformation**

Transgenic Arabidopsis was obtained with the same procedures and conditions as described in Chapter 1.

**Real-time PCR analysis**

Total RNA was isolated from seedlings (including both roots and above-ground portions), and FT transcripts were quantified by TaqMan real-time PCR (7500 Fast Real-time PCR system) using AtACT2 (GenBank Accession NM180280) for normalization as described in Chapter 1.
Results

Induced floral transition in transgenic Arabidopsis

To examine the potential of application of this system for the control of plant morphology, pSUM46-FT, in which the flowering locus T (FT) gene was replaced with the sGFP gene, was introduced into Arabidopsis via vacuum infiltration and 17 independent kanamycin-resistant transformants were obtained. Of these primary transformants, 10 transformants, which were expected to carry single-copy T-DNA, were selected by kanamycin-resistant segregation in the T$_2$ generation. Homozygous lines were obtained from these plants and 5 of the 10 lines were chosen at random for the floral transition test. Floral transitions were induced by copper in all lines tested (Fig. 2-1 (a), the results from line 46FT-2b). Ten-day-old seedlings grown under 12-hours light/12-hours dark conditions were transferred to agar medium with or without 100 $\mu$M CuSO$_4$. Because 1-day treatment of 100 $\mu$M CuSO$_4$ was insufficient for highly reproducible floral induction, seedlings treated with 100 $\mu$M CuSO$_4$ for 2 days were then retransferred to agar medium that did not contain CuSO$_4$. Visible floral buds were confirmed on the induced plants within 5 days of retransfer, whereas no visible floral buds were confirmed on the uninduced plants (Fig. 2-1 (a)). Although 1 of the 5 lines exhibited some early flowering in the absence of copper, the flowering time of the remaining lines was the same as for the wild-type under 12-hours light/12-hours dark conditions. On the other hand, transgenic Arabidopsis 35S-FT showed a strong early flowering phenotype with a flower bud that appeared on the top of skinny plants, as shown in Fig. 2-1 (a).

To further investigate, T$_3$ seeds of 46FT-2b were germinated on agar medium supplemented with 0, 5, or 10 $\mu$M CuSO$_4$. Seedlings were then grown under 12-hours light/12-hours dark conditions. During the vegetative phase, those plants grown on an agar medium with 10 $\mu$M CuSO$_4$ produced only 3.5 ± 1.0 rosette leaves, which was a similar number to that produced by 35S-FT plants (Table 2-1). In contrast, plants from line 46FT-2b grown without CuSO$_4$ produced 8.1 ± 2.4 rosette leaves, which was a similar number to that produced by wild-type plants. This means that 10 $\mu$M CuSO$_4$ was sufficient to induce floral transition when plants were sown on copper-containing medium and that background expression was practically negligible, even when plants were grown on agar medium containing 0.1 $\mu$M CuSO$_4$ that is contained in MS salts. In addition, plants from line 46FT-2b that were grown on agar medium with 5 $\mu$M CuSO$_4$ produced 5.0 ± 1.3 rosette leaves, which was more than were produced by plants grown on agar medium with 10 $\mu$M CuSO$_4$. In fact, FT transcripts in 46FT-2b seedlings grown on agar medium containing CuSO$_4$ for 10 days was induced in a dose-dependent manner and there was a greater amount of FT transcripts in 46FT-2b seedlings grown on agar medium containing 10 $\mu$M CuSO$_4$ for 10 days than in 35S-FT seedlings (Fig. 2-1 (b)).
(a) Ten-day-old seedlings of wild type (Col), line 46FT-2b, and 35S-FT grown under 12-hours light/12-hours dark conditions were transferred to agar medium that was (+) or was not (−) supplemented with 100 μM CuSO₄. After 2 days, the seedlings were retransferred to agar medium that did not contain 100 μM CuSO₄. Five days after retransfer, a bright-field image was obtained.

(b) Wild-type (Col), T₃ seeds of 46FT-2b and 35S-FT were germinated on agar medium supplemented with 0, 5, or 10 μM CuSO₄. Seedlings were then grown under 12-hours light/12-hours dark conditions. Total RNA was extracted from 10-day-old seedlings and analyzed using real-time PCR. The amount of sGFP mRNA and FT mRNA were normalized to that of actin. The bars indicate the SD of 3 experiments. 46FT-2b and 35S-FT transgenic plants were transformed with pSUM46-FT and pBI-35S-FT, respectively.

Fig. 2-1  Induced floral transition in transgenic Arabidopsis.
Table 2-1  Comparison of the number of leaves in transgenic *Arabidopsis* induced by copper at various concentrations

<table>
<thead>
<tr>
<th>Line</th>
<th>Copper (μM)</th>
<th>No. of rosette leaves*</th>
<th>No. of cauline leaves*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Col)</td>
<td>0</td>
<td>7.8±2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2±0.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.6±1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4±1.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8.0±2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.6±1.2</td>
</tr>
<tr>
<td>46FT-2b</td>
<td>0</td>
<td>8.1±2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3±0.7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.0±1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9±0.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.5±1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.9±0.8</td>
</tr>
<tr>
<td>35S-FT</td>
<td>0</td>
<td>3.6±1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2±1.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.7±1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.0±0.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.4±1.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.1±0.9</td>
</tr>
</tbody>
</table>

* The number of rosette leaves and cauline leaves were counted when flower buds appeared. Values are the means ± SE of 36 plants for each treatment. Different letters indicate significant differences (P < 0.05, Tukey-Kramer HSD test).
Discussion

For the precise investigation of gene function, two or more inducible systems should be used in order to distinguish the influences of inducers and the expression of transcription factors. Our newly developed copper-inducible system results in rapid up-regulation of target gene expression (within 6 hours) with relatively slow down-regulation. These data suggest that this system can be used for the induction of master key genes, such as the floral integrator $FT$, that require expression for only a short time in order to function. In such applications, prolonged exposure to high levels of copper is not required and copper toxicity would not be apparent. In fact, transgenic Arabidopsis carrying $pSUM46$-$FT$ that was treated with 100 μM copper for 2 days exhibited early flowering without any toxicity. Additionally, 10 days of treatment with 10 μM copper in germination medium was sufficient to induce floral transition, accompanied by a greater amount of $FT$ transcripts than is found in 35S-$FT$ seedlings. On the other hand, when compared with the amount of $FT$ transcripts in the wild-type, 46FT-2b seedlings that were grown on agar medium without CuSO$_4$ seemed to have high background expression of the $FT$ gene. However, I suppose this relatively high background expression simply reflects the difference between wild-type plants and 46FT-2b plants in the number of cells which express the $FT$ gene. Considering that the native $FT$ gene is specifically expressed in the vascular tissues of leaves, if an appropriate promoter for the ACE1/VP16AD expression is selected, more precise expression control could be achieved. Nonetheless, the timing of floral transition of 46FT-2b plants changed artificially by copper-induction. Our results demonstrated that our newly developed system could be a good tool for studies of development, circadian biology, and other basic studies.
Chapter 3

Application study to a new detection tool for bioavailable copper in soil

Introduction

Copper is an essential element for all known living organisms, including humans and other animals. Though necessary at low concentrations, it is potentially toxic at higher levels (Dorsey et al. 2004). Major anthropogenic copper emitters include smelters, iron foundries, and power stations. In addition, agricultural use of copper products like fertilizers and fungicides accounts for 2% of the copper released into soil (Howe et al. 1998). Most copper deposited in soil is strongly adsorbed to organic matter, carbonate minerals, clay minerals, hydrous iron, and manganese oxides. In fact, one report determined that the exchangeable fraction is less than 5% of the total copper concentration, even in rhizosphere soil (Brannvall et al. 2014). The uptake of copper by plants is highly dependent on the nature of the soil itself, as well as the pH, presence of oxides, redox potential, charged surfaces, organic matter, and cation exchange. In addition, soil microbes also affect uptake of copper (Wang et al. 2007). Nonetheless, most regulations are based only on the physico-chemical data of samples extracted from soil by hydrochloric or nitric acid. Though physico-chemical methods of detection are highly sensitive and accurate, they have limited applicability in the biological context, since they cannot distinguish between the total amount in the environment and the actual amount available for biological use (the bioavailable amount). Therefore, the development of simple and more sensitive bioassays for copper is needed to complement the existing physico-chemical techniques.

The potential use of the bacterium Achromobacter sp. AO22 as a copper biosensor has been previously described (Ng et al. 2012). This biosensor has a wide linear detection range (6–191 ppm), but the assay is far limited to a liquid suspension culture. Before its validation for environmental use, there are many challenging details to be investigated, including the stability of the strain, reproducibility of the assays, and ease of use. Seeking an alternative solution, I described in Chapter 1 that the successful development of a copper-inducible gene expression system utilizing transgenic plants, which is based on the regulation of a yeast metallothionein gene. This system’s advantages include: 1) copper-specific responsiveness; 2) dose-dependent responses; and 3) a dynamic range from 1 μM to 500 μM (0.06 ppm to 32 ppm (weight/volume)) at the transcriptional level in
an artificial system (agar medium supplemented with CuSO$_4$). Here I describe a more practical demonstration of this system, as a copper biosensor in soil.
Material and Methods

Plasmid construction

From Plasmid CaMV35S-sGFP(S65T)-NOS3', the TMV Ω sequence was amplified and then replaced with the To71 sequence of pSUM48-sGFP as described in Chapter 1, to create pSUM01-sGFP. pSUM02-sGFP included the XbaI site upstream of the TMVΩ sequence of pSUM01-sGFP. Sequence A94, the 5′-untranslated region sequence derived from an alcohol dehydrogenase gene of tobacco, was amplified from synthetic oligonucleotides (A94-1F, 5′-gtctattaactcagtattcagaaccaaaagttcttcctacataaattttctatattgtgatcagtgaagagaatcaagaaaaataaatg-3′ and A94-1RC, 5′-catttattttctgattttcactgactaatagaaatatattatatgtagagaagaactttgtgattctgaatactgagttaaatagac-3′), and replaced with the To71 sequence of pSUM48-sGFP to create pSUM03-sGFP. pSUM04-sGFP included the XbaI site upstream of the A94 sequence of pSUM03-sGFP.

Plant Transformation

Transgenic Arabidopsis was obtained with the same procedures and conditions as described in Chapter 1.

Copper treatments

For real-time PCR analysis, transgenic plants were transferred to agar medium that was or was not supplemented with 100 μM CuSO₄. For soil type screening, Aisai No.1 (Katakura Chikkarin), Black soil (Iwamoto), New powersoil (Kanto Hiryo), Potsoil tokugo, Potsoil for green onion (Honen Agri), Takii baido for onion (Takii seed), and Yosaku (Jcam Agri) were used. For GFP fluorescence microscopic detection, transgenic plants were transferred to 10 g of black soil (pH 6.0) in 6-well plates, and supplemented with 3.0 ml of one of various concentrations of CuSO₄ solution per well. In another case, 10 g of black soil, thoroughly premixed with various amounts of metallic copper (fine powder particle size <63 μm) and 3.0 ml of water, were used. The seedlings transferred to soil were incubated in a growth chamber at a temperature of 23°C and a humidity of 60% under a 23/1-h photoperiod at 65 μmol photons m⁻² sec⁻¹ for 3 days.
**Real-time PCR analysis**

Total RNA was isolated from seedlings (including both roots and above-ground portions), and sGFP transcripts were quantified by TaqMan real-time PCR (7500 Fast Real-time PCR system) using AtACT2 (GenBank Accession NM180280) for normalization as described in Chapter 1.

**Microscopy**

After 3 days of incubation with the copper treatments, fluorescent and bright-field images of plants were observed using a fluorescence microscope (VB-G05; Keyence, Osaka, Japan), and photographed using a ccd camera (VB-7010; Keyence, Osaka, Japan). The exposure time for fluorescent images was set to 1 sec.
Establishment of transgenic plants and confirmation of copper-induced gene expression

As described in Chapter 1, I have established a tightly regulated, highly responsive copper-inducible gene expression system. This system utilizes two elements: (1) a chimeric gene consisting of the transcription factor gene of yeast activating a copper-metallothionein gene (ACE1) fused to the VP16 activation domain (VP16AD) of the herpes simplex virus; and (2) a gene of interest that is under the control of a target promoter that includes 4 copies of MRE fused to 35S minimal promoter and the T071 sequence. The addition of VP16AD to ACE1 was the most effective in enhancing copper-induced gene expression. However, many applications using such a system are problematic unless basal expression is negligible in the absence of copper. In our previous study, I found the T071 sequence reduced basal expression of the reporter gene to negligible levels.

To confirm this effect, 4 more binary vectors were constructed by replacing the T071 sequence with either the Ω or A94 sequence (Fig. 3-1). The Ω sequence is very similar to T071, whereas the A94 sequence is not. Nonetheless, both are known as translational enhancers (Gallie et al. 1987, Satoh et al. 2004). In this study, when the GFP gene was used as a reporter gene, plant lines 01–04 (carrying the Ω or A94 sequences instead of T071) demonstrated strong GFP fluorescence in response to copper, similarly to plant line 48. Additionally, basal expression levels of lines 01–04 were as low as that of line 48 (Fig. 3-2). Investigating the possibility that the additional sequences (like the XbaI sequence) located upstream of such 5′-untranslated region sequences might affect induction efficiency, minor changes to transgenic plant lines 02 and 04 were made and evaluated (Fig. 3-1). However, there were no significant differences in basal or induced levels versus the line 01 and 03 plants. In Fig. 3-2, the 60 independent transgenic lines are sorted in ascending order based on basal sGFP transcript levels. There were variations in basal and induction levels of expression among independent lines rather than the difference of constructs. This is probably due to a positional and/or silencing effect. From all lines, 4 representative lines (01–11, 01–37, 46–6, 48–5) were selected for soil-based experiments, based on their good responses to copper combined with differences in their basal expression of the sGFP transcript. Notably, the agar medium that was used in this study normally contains 0.1 μM copper, as one of the MS salt microelements. Thus, basal expression may be increased even in controls by the copper from the MS salt. On the assumption of that, 4 representative lines were selected, and differences of these lines’ sensitivity to copper were examined.
The sGFP gene was inserted downstream of a target promoter that consisted of 4 copies of MRE fused to the 35S(-90m) or 35S(-46) minimal promoter. The To71, Ω, or A94 sequence was inserted between the minimal promoter and the sGFP gene. The amino acid sequence of the chimeric transcriptional activator consisted of the full length of ACE1 and the VP16 activation domain of the herpes simplex virus. pSUM46-sGFP, pSUM48-sGFP, pSUM01-sGFP, pSUM02-sGFP, pSUM03-sGFP, and pSUM04-sGFP are represented by 46, 48, 01, 02, 03, and 04, respectively. The symbol “*” indicates a mutation region of the ASF1 binding site in the 35S promoter.

35S pro, the CaMV 35S promoter;
ACE1, the transcription factor for the activating copper-metallothionein expression;
CDBD, the copper and DNA binding domain;
AD, the activation domain; MRE, the metal responsive element;
To71, the 5′-UTR sequence of RNA-dependent RNA polymerase gene of tomato mosaic virus;
Ω, the 5′-UTR sequence of RNA-dependent RNA polymerase gene of tobacco mosaic virus;
A94, the 5′-UTR sequence of an alcohol dehydrogenase gene of tobacco;
CR16 ter, the carrot CR16 terminator;
NOS ter, the NOS terminator;
Kanr, the kanamycin resistance gene.
Fig. 3-2  Comparison of sGFP transcript levels.

Six 10-day-old seedlings of T2 plants were transferred to agar medium that was (light gray columns) or was not (dark gray columns) supplemented with 100 μM CuSO4. Total RNA was extracted after 6 hours of incubation and analyzed by real-time PCR. Transgenic plants that were transformed with pSUM46-sGFP, pSUM48-sGFP, pSUM01-sGFP, pSUM02-sGFP, pSUM03-sGFP, and pSUM04-sGFP are represented by 46, 48, 01, 02, 03, and 04, respectively. Each line has 7-13 independent events. Lines 01–37, 46–6, 48–5, and 01–11 shown in the meshed box were used in subsequent experiments.

**Different responses to copper in soil**

Before copper treatment experiments, 7 types of soil were screened using 14-day-old seedlings of T3 plants (line 48–5). Some plants demonstrated GFP fluorescence after being transferred to particular soil types even without copper supplementation, indicating the presence of copper in the basic soil and disqualifying that soil from use here. In addition, one soil was not appropriate for growing *Arabidopsis thaliana*. Thus, black soil was selected for use, as the 48–5 line plants showed no baseline GFP fluorescence or inhibition of their growth.

To test copper uptake from the soil by the plants, two types of copper were used, CuSO4 solution and metallic copper (as powder premixed in the soil). I assumed that the amount of premixed metallic copper was equal to the total copper in the soil. In fact, when the line 01–11 plants were transferred to soil and supplemented with
CuSO₄ solution, weak GFP fluorescence was observed in a portion of the leaves at 0.4 ppm, and stronger GFP fluorescence was observed in a larger number of leaves with increasing copper concentrations (Fig. 3-3). GFP fluorescence suggestive of spreading of the copper was observed along the veins at 1 or 2 ppm, and at 10 ppm, very strong GFP fluorescence was observed in all leaves except cotyledons. However, when the line 01–11 plants were transferred to soil premixed with metallic copper, GFP fluorescence was observed beginning at 20 ppm, with GFP fluorescence strengthening with increasing copper concentrations from 20 ppm to 500 ppm. These results indicated that uptake of copper when supplemented with CuSO₄ solution is 50 times easier than that when supplemented metallic copper, and that the transgenic plants respond selectively to bioavailable copper. Because of the inherent variations in basal and induced expression, the other transgenic lines can be selected for their application-specific attributes. Indeed, when line 48–5 was used instead of line 01–11, the detection limit was 1 ppm of CuSO₄ and 50 ppm of metallic copper (Fig. 3-4); and when lines 46–6 or 01–37 were used, it was 4 ppm of CuSO₄ and 200 ppm of metallic copper, respectively (Fig. 3-5, 6). The detection limit of line 46–6 was the same as that of line 01–37, but the intensity of GFP fluorescence appeared stronger than that in line 01–37. These differences in sensitivity were correlated to the basal expression levels of the sGFP transcript (Table 3-1). At the higher concentrations of copper (e.g., 10 ppm of CuSO₄ or 500 ppm of metallic copper), the distribution of GFP fluorescence was not quite different among these tested lines when compared to that at the lower concentrations (Fig. 3-3, 4, 5, 6). This convergent effect was correlated with the similarity of induced sGFP transcript expression levels at the higher concentration (Fig. 3-2). Meanwhile, the significant differences in GFP fluorescence at the lower concentrations could be amplified by the difference between the half-lives of the mRNA (sGFP transcript) and the protein (GFP). Given these differences, a line whose bioavailable copper sensitivity falls within the application’s optimal target range could be selected, preventing false positive or negative responses related to excessive or inadequate detector sensitivity.
Fig. 3-3  Induced GFP expression in line 01–11 by copper in soil.

Fourteen-day-old seedlings of T₃ plants were transferred to soil and (a) supplemented with 10, 20, 50, 100, 200, 500 µM CuSO₄ solution in each well (0.2, 0.4, 1, 2, 4, 10 ppm (weight of copper/weight of soil)), or (b) transferred to soil premixed with 10, 20, 50, 100, 200, 500 ppm metallic copper (powder) and supplemented with water. Fluorescent (upper panels) and bright-field (lower panels) images were obtained after 3 days of incubation.
**Fig. 3-4** Induced GFP expression in line 48–5 by copper in soil.

All the pictures in Fig. 3-4 were obtained with the same procedures and conditions as Fig. 3-3.
Fig. 3-5  Induced GFP expression in line 46–6 by copper in soil.

All the pictures in Fig. 3-5 were obtained with the same procedures and conditions as Fig. 3-3.
Fig. 3-6  Induced GFP expression in line 01–37 by copper in soil.

All the pictures in Fig. 3-6 were obtained with the same procedures and conditions as Fig. 3-3.

Table 3-1  Correlation between basal expression level and detection limit

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>Relative $sGFP$ transcript level at 0.1μM CuSO$_4$</th>
<th>CuSO$_4$ Detection limit (ppm)</th>
<th>Metallic copper (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01–37</td>
<td>0.3</td>
<td>4.0</td>
<td>200</td>
</tr>
<tr>
<td>46–6</td>
<td>1.0</td>
<td>4.0</td>
<td>200</td>
</tr>
<tr>
<td>48–5</td>
<td>1.7</td>
<td>1.0</td>
<td>50</td>
</tr>
<tr>
<td>01–11</td>
<td>3.1</td>
<td>0.4</td>
<td>20</td>
</tr>
</tbody>
</table>
Discussion

By utilizing transgenic plants carrying a copper-inducible reporter gene expression system, bioavailable copper in the soil could be assessed. If other reporter genes such as firefly luciferase (LUC) or β-glucuronidase (GUS) were used in place of GFP, quantification of bioavailable copper might be easier and more accurate. However, the GFP gene is adequate, especially for pre-screening purposes, since GFP fluorescence is easy to detect, and its intensity and distribution correlated with the concentration of bioavailable copper. Establishing the relationship between copper content and GFP fluorescence by measuring the copper content in the plant in advance would provide surer footing for the use of this system. In addition, since bioavailable copper concentrations can be affected by pH, this system would be more useful and exploited in various fields if further investigation finds it can also detect differences under different pH conditions.

Copper concentrations are typically 14–29 ppm dry weight for surface soils, and <50 ppm for sediments (Dorsey et al. 2004). The guidelines for copper levels in a Japanese rice field are set at 125 ppm (https://www.env.go.jp/kijun/dt1.html), while A&L Eastern Laboratories, Inc. (Virginia, USA) describes copper levels in soil that may be unsafe for leafy or root vegetables to be >200 ppm, and for gardens or child exposure >500 ppm (http://www.aleastern.com/forms/Heavy%20Metal%20Interpretation.pdf). The transgenic plants used in this study are adequate for detection at these concentration ranges. Thus, they provide a convenient and beneficial tool for pre-screening of samples, as well as a solid, biologically relevant complement to the physico-chemical methods of copper assessment.

DNA microarrays have been used to detect the effects of soil contaminants on plant gene expression (Magrini et al. 2008). While this might be useful to capture the broader effects on the plant transcriptome, it required many steps and was difficult to interpret correctly in some cases. Even when compared to microbe-based assays (Ng SP et al. 2012), this transgenic plant-based system is simpler and less expensive because extraction and purification steps, and reagents are not required. One advantage to microbe biosensor assays is their diversity, in that microbe biosensors for Cd, Pb, and other heavy metals have already been developed (Shetty et al. 2003, Liao et al. 2006, Diels et al. 2009). If essential components to detect such heavy metals can be genetically introduced into plants, such transgenic plants would also become considerably useful tools in this field.
Concluding Remarks

In the chapter 1, I described the newly developed copper-inducible gene expression system showed good performance in a tightly regulated and highly responsive manner. This study indicates that the addition of VP16AD to ACE1 was critical to the function of the copper-inducible system in plants, different from in yeast. In addition, interestingly, insertion of the To71 sequence, a 5′-untranslated region of the 130k/180k gene of tomato mosaic virus, upstream of the GFP gene reduced the basal expression of GFP in the absence of copper to almost negligible levels. These modifications enabled to regulate floral transition by copper treatment (chapter 2). Since exposure of plants to 100 μM copper resulted in an over 1,000-fold induction ratio at the transcriptional level of GFP, and this induction was copper-specific and dose-dependent with rapid and reversible responses, when the transgenic plants were transferred to soil, GFP fluorescence was observed in accordance with the content of bioavailable copper (chapter 3).

Establishing the relationship between copper content and GFP fluorescence by measuring the copper content in the plant in advance would provide surer footing for the use of this system. In addition, since bioavailable copper concentrations can be affected by pH, this system would be more useful and exploited in various fields if further investigation finds it can also detect differences under different pH conditions. However, this study successfully demonstrates a new transgenic plant-based system is able to detect bioavailable copper without any extraction and purification steps. In addition, these results indicate that the newly developed copper-inducible system can accelerate gene functional analysis in model plants and can be used to generate novel agronomic traits in crop species.
References


environmental applications with Cupriavidus metallidurans. Antonie Van Leeuwenhoek 96:247-258


Biol 17:1050-1054


Mett VL, Lochhead LP, Reynolds PH (1993) Copper-controllable gene expression system


Yamamoto M, Torikai S, Oeda K (1997) A major root protein of carrots with high
homology to intracellular pathogenesis-related (PR) proteins and pollen allergens.

Plant Cell Physiol 38:1080-1086


Acknowledgements

This dissertation is the outcome of my research in the doctoral course of Graduate School of Fisheries Science and Environmental Studies, Nagasaki University. I do express my deepest gratitude to Professor Dr. Yoshie Kitamura, for her very kind help, sincere guidance, generous advise, constant encouragement and continuous supervision as the Principal Investigator in conducting this research study and preparation of this dissertation. I would like to express my gratitude and sincere thanks to Dr. Takayuki Miyanishi, Dr. Masaya Nishiyama and Dr. Hideki Nakayama for their kind, valuable suggestion in preparing this dissertation.

I am grateful to Dr. Y. Niwa of Shizuoka Prefecture University for the kind gift of the 35S-sGFP(S65T) vector, and Dr. M. Mori of Ishikawa Prefecture University for the kind gift of piLerG3. I thank Team leader A. Nagasawa, Dr. R. Sato and Dr. T. Adachi in Sumitomo Chemical Co. for valuable discussions and suggestions, and Ms. T. Chikahisa, Ms. T. Nakamura, Ms. M. Uda, Ms. E. Tanaka, Ms. H. Hagita, Mr. M Toya, and Mr. R. Fukui in Sumitomo Chemical Co. for their technical assistance.