Trojan Horse at New Ilion Gate —CPPo: from cell to ´real plant´

Zur Erlangung des akademischen Grades eines

DOKTORS DER NATURWISSENSCHAFTEN
(Dr. rer. nat.)

Fakultät für Chemie und Biowissenschaften

Karlsruher Institut für Technologie (KIT) – Universitätsbereich
genehmigte

DISSERTATION

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Tag der mündlichen Prüfung: 16. 10. 2017

τῇ περ δὴ καὶ ἔπειτα τελευτήσεσθαι ἔμελλεν·
αἶσα γὰρ ἦν ἀπολέσθαι, ἐπὴν πόλις ἀμφικαλύψῃ
δουράτεον μέγαν ἵππον, ὅθ᾽ ἅτο πάντες ἄριστοι
Ἀργείων Τρώεσσι φόνον καὶ κῆρα φέροντες.

"for it was their fate to perish when their city
should enclose the great horse of wood,
wherein were sitting all the best of the Argives,
bearing to the Trojans death and fate"

———*Homer Odyssey VIII 512-514*

Die vorliegende Dissertation wurde am Botanischen Institut des Karlsruher Instituts für Technologie (KIT), Botanisches Institut, für Molekulare Zellbiologe, im Zeitraum von September 2013 bis Juli 2017 angefertigt.

Hiermit erkläre ich, dass ich die vorliegende Dissertation, abgesehen von der Benutzung der angegebenen Hilfsmittel, selbständig verfasst habe.

Alle Stellen, die gemäß Wortlaut oder Inhalt aus anderen Arbeiten entnommen sind, wurden durch Angabe der Quelle als Entlehnungen kenntlich gemacht.

Diese Dissertation liegt in gleicher oder ähnlicher Form keiner anderen Prüfungsbehörde vor.

Karlsruhe, 2017

LIANG Ye

Acknowledgement

In the four years PhD study, I have received numerous helps and kindness from many people. Here, I want to give my sincere gratitude to those who have helped and cared about me, and I want to say I could not finish my PhD period without you.

Firstly, to Prof. Dr. Peter Nick, a simple Danke Schön could not express how gratitude I am to his help and guidance. As he often says, life is not easy. In this harsh world and these challenging years, more than once he gives me his helping hands not only in scientific issues but also in my daily life. I could not ask for a better Doktorvater.

Secondly, I would like to say thank you to Prof. Dr. Ute Schepers (Institute of Toxicity and Genetics, Karlsruhe Institute of Technology, Campus North, Karlsruhe, Germany). As one pillar of this interdisciplinary research, she kindly sys provides all CPPo used in this research, including DOK848 and the whole CPPo library. She has enlightened me in chemistry aspect of peptoids, and how these peptoids would behave in animal organ level.

Colleagues and friends in Botanic Institute I have supported me a lot. We work together, we laugh together. They treat me as family member and I consider them as my brothers and sisters. Among them, Dr. Jan Maisch gives me a lot help on the microscopy techniques and cell subculture techniques, Dr. Michael Riemann gives me advices on rice cultivation techniques and rice seed germination, Dr. Liu Qiong helps me a lot on everyday wet bench work. I really appreciate their kindness and help.

My parents have shown great understanding and support in last four years. I have not travel back to China once, and they did not blame me. Their visit in 2016 lighted my days. I love you, mom and dad.

As for my better half, thanks for company me during these stressful days in my life. Every bit of love’s joy and woe gives me strength that hold me through these days, and loose some bodyweight. May we company each other in the years to come.

This work has supported by China Scholarship Council (CSC).

At last, I would like to give my thanks to all the cells and plants I have been working on in this research. It is you show me the beauty of science, and how magnificent plant life is.

Karlsruhe, 2017

LIANG Ye

Zusammenfassung

Molekulare Transporter wurden entdeckt, synthetisiert und in biologischen und medizinischen Bereichen eingesetzt. Zellpenetrierende Peptoide (CPPo) gehören zu den vielversprechendsten molekularen Transportern. Doch bevor CPPo in der wissenschaftlichen Forschung und im klinischen Bereich eingesetzt werden können, müssen zwei Hauptprobleme gelöst werden. Zuerst sollte die CPPo-Verteilung in Zielorganismen untersucht werden. Zweitens müssen CPPo mit dem Frachtmolekül verknüpft werden, um zu einem biofunktionalen Partikel zu werden bzw. das Frachtmolekül sollte in den untersuchten Organismen wie erwartet funktionieren.

Im Rahmen dieser Doktorarbeit wurde eine Bibliothek von CPPo sowohl im Suspensionszellsystem Tabak BY-2 als auch im pflanzlichen System Reis Dongjin getestet. Zuerst wurde die Membranpenetrationfähigkeit einzelner Mitglieder dieser CCPo-Bibliothek getestet, sodass neununddreißig Kandidaten aufgrund ihrer Penetrationsfähigkeiten in Suspensionszellen als geeignet für weitere Studien angesehen wurden. Zweitens wurden die Verteilungsmuster aller CPPo in dieser Bibliothek sowohl in Suspensionszellen als auch in Reiswurzelspitzen untersucht und insgesamt drei Verteilungsmuster auf der Zellebene bzw. zwei Musterauf der Ebene pflanzlicher Gewebe gefunden.

Um die Penetrationsmechanismen und die Musterbildungsmechanismen zu verstehen, wurden mehrere Hemmstoffe verwendet, um die Penetrations- und Musterbildungsprozesse von ausgewählten CPPo-Kandidaten zu blockieren. Als Hemmstoffe wurden Latrunculin B, Oryzalin und BDM verwendet. Allerdings wurden keine spezifischen Mechanismen gefunden.

Schließlich wurde ein praktisches Anwendungsbeispiel mit dem CPPo DOK848 durchgeführt. DOK848, ein mit Colchicin gekoppeltes CPPo, wurde in einer BY-2 Mikrotubulimarkerlinie im Vergleich mit Colchicin getestet. DOK848 konnte erfolgreich Colchicin in BY-2-Zellen einschleusen und in deutlich geringeren Konzentrationen eingesetzt werden als es bei alleiniger Colchicingabe der Fall war.

Abstract

Molecular transporters have been found, synthesised and used in biology and medical fields. Cell penetrating peptoids are one of those promising ones. However, before CPPo can be used in scientific research and clinic field, two major problems must be solved. First, CPPo distribution in target organisms should be studied. Second, CPPo could linked to the cargo molecule and become a biofuctionalized particle, and the cargo molecule should function inside organisms as expected.

Within the scope of this research, a library of CPPo has been tested in both suspension cell system which is tobacco BY-2 cells and plant system which is rice Dongjin cultivar. First, membrane penetration ability of individual members in this CCPo library had been put to test, thirty nine candidates are considered to be most suitable for further studies due to their penetration abilities inside suspension plant cells. Second, the distribution patterns of all CPPo in this library have been found in both suspension cells and rice root tips, totally three patterns in cell level and two patterns in tissue level.

In order to understand the penetration mechanisms and pattern forming mechanism, several bio-blockers have been used to block the penetration and pattern forming process of selected CPPo candidates. Blockers are including latrunculin B, Oryzalin, and BDM, etc. However, none specific mechanism has been found.

Last, one CPPo practical application example. DOK848, a colchicine loaded CPPo, has been tested in a microtubule labeled BY-2 line side by side with colchicine. DOK848 has successfully transfer colchicine into BY-2 cells meanwhile the cargo colchicine could function as it is in animal cells, while the normal colchicine needs much higher concentration to penetration into BY-2 cells.

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List of abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
BDM	2,3-butanedione monoxime
BY-2	*Nicotiana tabacum* L.cv. Bright Yellow 2
CPP	Cell-Penetrating Peptide/ Cell-Permeating Peptide
CPPo	Cell-Penetrating Peptoid/ Cell-Permeating Pepotid
DMSO	Dimethyl sulfoxide
GFP	Green fluorescent protein
LatB	Latrunculin B
NLS	Nuclear localization sequence
PTD	Protein transduction domains
RFP	Red fluorescent protein

1 Introduction

Transportation and means of transport have played an important role in human's history. From using cattle to carry working tools to farmland to sending cargos into orbit and Earth' nearby satellite by space ship, from delivering hoplites into hostile city in minor Asia by a huge wooden horse to releasing drugs in specific organs and cells, mankind have invented many ways to transport target cargo to a certain destination. Precision, fast, convenient to use and economic are the standard for a good means of transportation. In modern biology and medical research field, scientist always need all kinds of methods and tools to deliver certain chemicals to a certain part of organisms. Meanwhile, the cargo chemicals should maintain the original biological or clinical function and perform normally. In this research, a library of Cell Penetrating Peptoids have been put into test to show their potential in botany research. But before we go into our topic cell penetration peptoids, a brief review of how this kind of chemicals have been designed is necessary. First, a look of its origin: cell penetrating peptide.

1.1 A short history of CPP

1.1.1 What is CPP

Three decades ago, a kind of newly discover chemicals became a hot topic in both chemistry and biology science community. These peptide containing only several short amino acid residues could translocate across plasma membranes with out any receptor mediated (Langel, 2002). Because of this unique trait, they are give a name: cell penetrating peptide, or cell permeating peptide, CPP for short.

In 1988, two groups American scientists found that Tat protein could penetrate into cells after been added to culture media (Green M, et al., 1988 and Frank A D, et al., 1988). Tat protein is a regulatory protein in coded in HIV, it has been well studied by virologists because it is essential for HIV replication (Dayton A I, et al., 1986). It has 86 amino acids residues and binds to the viral TAR region, it also could increase the processivity of RNA polymerase and transactivates the viral promoter (Jeang KT, et al., 1999). However, a series of studies show that the whole sequences of Tat protein is not necessary for its

invasion ability. A recombinant protein containing β-galactosidase and a part of Tat peptide that could be uptake by many cell lines had been synthesised (Fawell, et al., 1994) . This protein encompasses the amino acids 37 to 72 in Tat protein. One year earlier, a shorter Tat peptide containing amino acids from 37 to 62 successfully transport a Fab antibody fragment into cells (Anderson D C, et al. 1993). In 1995, it is found that by biotinylation of Tat protein the uptake of full length Tat protein would increase (Chen L L, et al. 1995).

One year later, another cell penetrating protein from insect has been found. Derossi et al. described the ability of the *Drosophila* Antennapedia protein's homeodomain that could transmembrane in a non-receptor fashion (Derossi D, et al. 1996). And the chemical structure responsible for this trait is an amphipathic 16 amino acids long region in the homeodomain third helix (Derossi D, et al. 1994).

Based on these initial findings, two engineer rule based on Tat protein were established in order to search for the shortest cell penetrating peptide derived from Tat protein (Sabatier J-M, et al. 1991 Perez A, et al. 2001) : 1. To reduce the cost, a CPP vector should be as short as possible and 2. Biology activities such as toxicity and transactivation properties that long Tat-derived peptides have should be avoided. And for now, the second guideline should be: A CPP or CPPo derived from other proteins or peptides should avoid any side effects caused by the origins' biology activities.

Table 1.1 shows that the short peptides derived from Tat protein and it relative cellular internalization efficacy. From this table it could tell that the shortest sequence and the higher signal corresponds to a 9-mer basic amino acids rich sequence Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg. This short sequence was strictly requested and by it appearance it is sufficient for the peptide be taken up by cells. More interestingly, it could accumulate in nuclei, showing that this peptide not only have a plasma membrane translocation ability but also contains a nuclear locatlization signal domain (Vivès and Lebleu 2002).

These initial studies draw a promising future of Tat protein and its derivatives' potential utilization as vehicles to transport cargoes into cells. And such molecular transporter are urgently needly in medical field. Traditionally, polypeptides and oligonucleotides are thought not suit for therapeutical utilization due to the low plasma permeability and fast degradation. But with the finding of CPP, this situation changed.

Primary sequence of the Tat peptides	Relative uptake efficacy
FITKALGISYGRKKRRQRRRPPQC	+
LGISYGRKKRRQRRRPPQC	++
FITKALGISYGRKKRRQC	-
GRKKRRQRRRPPQC	++++
GRKKRRQRRRC	++++
GRKKRRQRARPPQC	++
GRKKRRQARAPPQC	+
GRKKRRQRRPPQC	++
GRKKRRQRPPQC	+/-
GRKKRRQPPQC	-
RKKRRQRRR	+++++
RRRQRRKKR	++++++
RRRQRRKKR	++++++

Table 1.1 Primary structure of the synthesized Tat peptide and structure activity study of the uptake (Vivès and Lebleu 2002)
The peptides uptake efficiency in this table were evaluated by the mean intensity of the fluorescent signal observed by microscopy or by FACS.

1.1.2 The classes of CPP

Except Tat protein and its derivates, some nature proteins have similar penetration abilities have been discovered. Such as Antennapedia of *Drosophila*, VP22 of HSV (protein transduction domains, PTD). Later derivates has been designed and synthesised such as MTS-NLS and transportan, model peptides and other PTDs.These CPP and their derivates enriched the arsenal that could be applied for cellular delivery of cargoes.

In table 1.2, Tat fragment (Shen W C, et at., 1979), penetratin (Shen W C, et al., 1978), signal sequence-based peptides (Bayard B, et al., 1986) and pVEC (Wagner E, et al., 1990) are proteins exists naturally, and amphiphilic model peptide (Curiel D T. 1994), transportan (Dayton A I. 1986) and Arg_9 (Jeang K T, et al. 1999) are artificial peptides.

Tat fragment and penetratin have been described above. Signal sequence-base CPP are main designed base on the nuclear localization sequences (NLSs) so they could penetrate

in through two barriers inside eukaryotic: cell membrane and nuclear membrane (Morris M C. 2000, Schwartz J. 2000, Mahato R I. 1999). Transportan derived from galparan which comprised from neuropeptide and a wasp venom peptide mastoparan (Langel et al. 1996). It is a 27-amino-acid-long peptide, and could penetrates in cells at physiological and low temperature without any endocytosis or active transport process (Pooga M, et al. 1998). Model amphiphilic peptides are a group of peptides designed by Steiner (Steiner V, et al. 1991) base on α-helical amphipathic model peptide KLALKLALKALKAALKLA-NH_2. They have been proved to be able to take up by mammalian cells in a nonendocytic fashion. The arginine-rich molecular transporters are based on the fact that oligomers of arginine composed of six amino acids or more could penetrate cell membrane very effectively alone or carry a small molecule (Mitchell D J. 2000). However the penetration mechanism is still unclear.

CPP classes	Original sequences for different CPP classes
Tat fragment (48-60)	GRKKRRQRRRPPQC
Penetratin	RQIKWFQNRRMKWKK
Signal sequence-based peptides	GALFLGWLGAAGSTMGAWSQPKKKRKV
Transportan	GWTLNSAGYLLKINLKALAALAKKIL
Amphiphilic model peptide	KLALKLALKALKAALKLA
pVEC	LLIILRRRIRKQAHAHSK
Arg_9	RRRRRRRRR

Table 1.2 Differnet CPP classes and their core sequences (Langel 2002)

1.1.3 The mechanisms of CPP cell penetration process

Since 1988 first CPP Tat protein was found, scientists started to look for the reason why these protein could invade in cells through the cell membrane. People use to believe that the penetration mechanisms of CPP (though it could be different among themselves) are different from those peptides used in clinical therapies to perform drug delivery such as CPP does not need receptors or by endocytosis or go through pores on the membrane.However the role of endocytosis in the uptake of CPPs have been found (Thorén, P.E.G. 2003, Richard, J.P. 2003). And endocytosis happens at the same time as

direct translocation across the plasma membrane (Guterstam P, et al. 2009). Receptor-dependent entry process has also been discovered (Ezzat K, et al. 2012) .

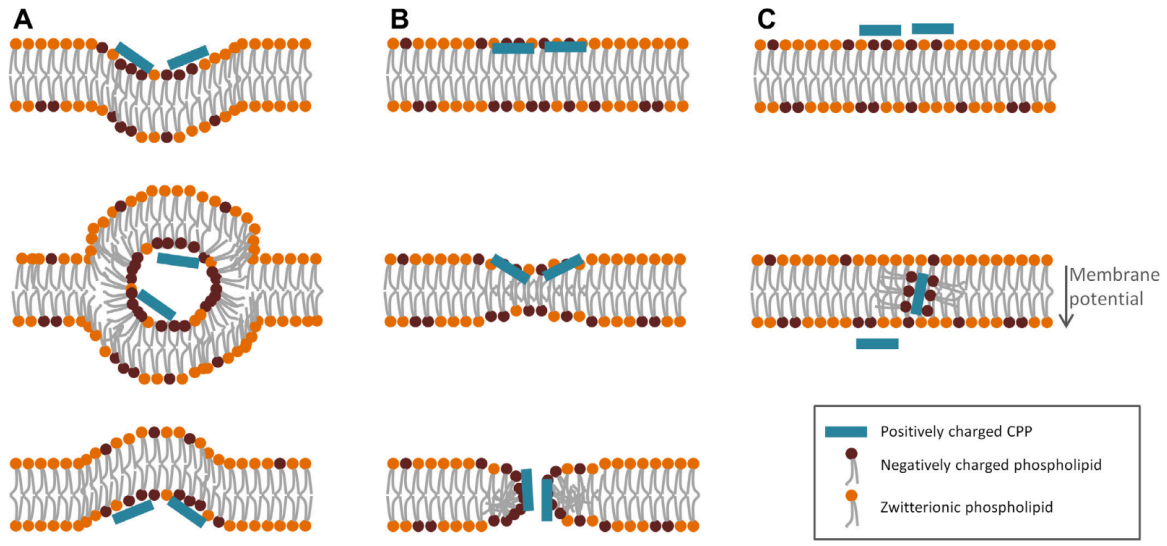


Figure 1.1 Some proposed mechanism of CPP translocate.
A is inverted micelle formation, B is pore -formation, C is adaptive translocation
(Bechara C. et al. 2013)

Though it is hard to illustrated a clear picture of how CPP goes into cells, but through study the chemical structure and penetration efficiency relationship, some general rules for CPP begin to emerge. Such as positive charges in side chains of lysine and arginine, amphiphilicity of the peptide, presence of tryptophan or phenylalanine in certain positions and the length of the polypeptide chain (Langel, 2002).

1.1.4 The applications of CPP

With the ability to permeate through the biological membranes, CPP changed the traditional views about peptides utilization.

From table 1.3 it could tell that CPPs have already been tested as carriers to bring cargoes into cells. The most well studied Tat protein and its derivates could carry peptide (Polyakov V, et al. 2000, Gius D R, et al. 1999), proteins (Fawell S, et al. 1994, Pepinsky R B, et al. 1994， Schwarze S R, et al. 1999, Vocero-Akbani A, et al. 2000, Xia H, et al. 2001), oligonucleotide (Astriab-Fisher A, et al. 2000), DNA (Eguchi A, et al. 2001), fluorescence

tag (Vivés E, et al. 1997, Bhorade R, et al. 2000), chelator (Bhorade R, et al. 2000), liposome (Torchilin V P, et al. 2001), particles (Lewin M, et al. 2000, Josephson L, et al. 1999). The antennapedia has delivered also peptides (Theodore L. 1995, Lindgren M, et al. 2000), protein (Han K, et al. 2000) and oligonucleotide (Troy C M, et al.1996, Allinguant B, et al. 1995). Artificially designed and synthesised Transportan has been used to transport peptide (Lindgren M, et al. 2000) and proteins (Pooga M, et al. 2001) into cells. Oligoarginine has carried Fluorescence tag (Wender P A, et al. 2000, Mitchell D J, et al. 2000, Futaki S, et al. 2001) and Peptide (Rothbard J B, et al. 2000). Loligomer,

CPP	**Cargos**
Tat	Peptide
	Protein
	Oligonucleotide
	DNA
	Fluorescence tag
	Chelator
	Liposome
	Particles
Antennapedia	Peptide
	Protein
	Oligonucleotide
	PNA
Transportan	Peptide
	Protein
	PNA
Loligomer	Fluorescence tag
	Plasmid DNA
	Drug
Hydrophobic signal	Peptide
	Protein
	Oligonucleotide
Oligoarginine	Fluorescence tag
	Peptide
VP-22	Protein

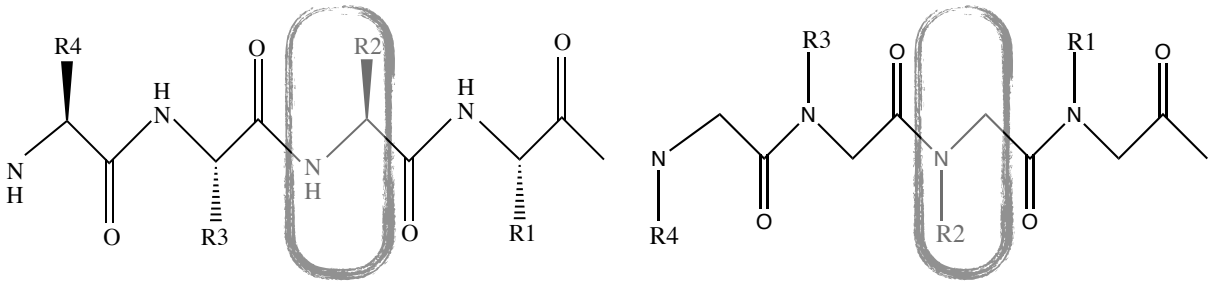
Table 1.3 Some CPP-assistance delivery systems (Tung C-H, 2002)hydrophobic signal and VP-22 these CPP which have not described above also been put into utilization.

After three decades development, more and more CPP are found or synthesised and been researched for their chemical and biological properties. Among them, a constantly growing number have been applied for bioactive cargoes delivery. There is no strict size limit of cargo has been found, therefore a bright future of application waits for CPP.

1.2 What is CPPo

1.2.1 The differences between CPP and CPPo

Although the CPP have been put to use in many aspects, it could not beat protease which exist in all kinds of biological environment. Thus, a kind of chemical mimics resist to protease degradation has been designed and synthesised. By simply moving the side chain form α-atom to nitrogen atom (seen in circles in figure1.2) , peptoids become protease resistance (Miller S M, 1995).



peptide peptoid

Figure 1.2 The structural differences between peptide and peptoid
In the grey circles the differences between peptide bond and peptoid bond are mark out

1.2.2 CPPo Traits

Besides its obvious advantages compare to peptide: highly stable against enzymatic degradation, CPPo also have many traits. Chemically, it has an chiral backbone and normally will not form secondary structure like peptide will (Kölmel D.K. et al, 2014). But by introducing suitable side chains peptoids could form a stable secondary even tertiary structures such as helics(Murnen H.K., 2010) , sheets(Nam K.T. et al,2010), threaded

loops(Huang K, et al, 2006). It is also much more easier to synthesis compare to other peptidomimetics (Kölmel D.K. et al, 2014).

In cell penetrating related area, CPPos always contain a lot of lysine- and/or arginine- like side chains. It is quite similar to R_9 class CPP. CPPos have already successfully transported siRNA (Utku Y, et al. 2006), photosensitizers (Lee J, et al. 2010), diverse fluorescence dyes (Kölmel D.K. et al. 2013) and lanthanide ions (Kölmel D.K. et al. 2013).

1.3 Scope of dissertation

The scope of this work can be basically divided into two major questions: 1. Do CPPo have biology function or biology function potential in suspension cell systems? 2. Do CPPo have biology function or biology function potential in real plant?

1.3.1 Do CPPo have biology function or biology function potential in suspension cell systems?

In plant science field, although we study varies types of plants, but most of physiology, genetics and cell biology studies are done in a handful of model plants or model systems, such as *Arabidopsis thaliana*, *Oryza sativa*, tobacco BY-2 cells, etc. Therefore, as an engineering oriented project, the potential of CPPo in future plant science research should be tested.

In order to search for the answer of this question, several small questions could be asked first.

1. Can CPPo in this library penetrate into plant cells?

This library was synthesised by a group in ITG leading by Prof. Dr. Ute Schepers. In her group, they were tested on Zebra fish. However, this library was never tested on any plant systems. Therefore, tobacco BY-2 suspension cell system has been selected as in vitro system to test CPPo, for BY-2 suspension cell system has a clear back ground because they has been widely used in plant studies. And compare to any other real plant model systems, it could be directly expose to CPPo, meanwhile the environments (both in vivo and in vitro) for them to grow are much more easier to control. The approach starts from

incubate BY-2 cells with CPPo. Preliminary experiments based on different parameters such as incubation time, CPPo concentration, incubation platform (erlenmeyer flask, epic tube or 24 well plate), shaking platform (horizontal shaker or rotor), shaking speed are done. The optimised assay setting is described in 2.5.2.

2. What is the behaviour of this CPPo library inside plant cells?

This general question could be replaced by some more specific questions. For example, are CPPo in this library toxic to plant cells? Could they locate in certain places or on some organelles? etc. Different answers to these questions can lead to different fate of this library's application in plant science field. To answer these questions, a massive screen assay for all the CPPo in BY-2 cells was designed and carried out. Using the spinning disc microscope, the behaviour of CPPo library could be clearly identified in microscopy pictures.

3. What is the mechanism of CPPo penetrating plant cells?

Although CPPo and CPP have been researched for more than a decade, but the mechanism of their penetration is still unclear. Now the common knowledge on this topic is: the CPP and CPPo's membrane translocation process is in a non-receptor mediated fashion, most of CPP and CPPo carry positive charge in physiology environment, and contain lysine and arginine residues, etc. But it is believed that maybe no common membrane translocate mechanism exists among CPP and CPPo (Langel, 2002). Every single kind of CPP and CPPo should be investigated for its own mechanism.

To achieved this goal, several inhibitors and blockers were put to use in this research as described in 2.4.

1.3.2 Do CPPo have biology function or biology function potential in real plant system?

A similar question can be asked after finding answers to the last question. So similar approaches has been put to use except BY-2 suspension cell system has been changed by a rice seedling system. When germination happens, root is the first organ that breaks the husk of seed, and it is also the first organ that allow plant to exchange material with

environment. Meanwhile the meristem determines the tissue differentiation in root. It means that meristem is a perfect target area for chemical engineering. Based on these reasons, rice root tip has been selected as the model system in this research to search.

In order to find out whether CPPo have a distribution pattern or favourite location in rice seedling root tip, rice seedlings were incubated with CPPo and then be observed under confocal microscope. The detailed methods are been described in 2.5.3.

1.3.3 A glimpse to the future application of CPPo

When finish looking through the properties of the vehicle, transportation with cargo should be tested in field. DOK848, also been synthesised in Prof. Schepers group, is such a cargo loaded vehicle. Its specific information is in 5.2. By analysing the entry efficiency and the working concentration of the loaded colchicine, DOK848 proved to be a efficient tool. And as a principle prove example, it shows that CPPo could become widely use in future.

2 Material and Methods

2.1 Tobacco cell cultures

2.1.1 BY-2 cell lines

Transgenic and non transgenic tobacco cell lines BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) have been put to applications in this research. For CPPo screening, distribution experiments etc, non transgenic tobacco cell line (marked as WT, wild type) have been used. For other experiments, two transgenic lines have been used, including: Tuβ6 line and GF11 line. Tuβ6 line could stably expressing a fusion construct of *Arabidopsis thaliana* β-6 tubulin (Chu B, et al., 1993) with GFP **,** and GF11 line could stably expressing a fusion construct of the second actin binding domain (ABD2) of *Arabidopsisi thaliana* AtFim1 (Sano et al., 2005) with GFP.

2.1.2 Cultivation of cell lines

All the tobacco BY-2 cell lines have been cultivated in liquid medium containing 4.3 g/L Murashige and Skoog salts (Duchefa, Haarlem, The Netherlands) which provides the major and minor elements in the medium, 200 mg/L KH2PO4, 100 mg/L inositol, 1 mg/L thiamine, 0.2 mg/L 2,4-D and 30 g/L sucrose. The pH of medium is 5.8. Every cell line was subcultured weekly. For WT cells, 1 mL cells were transferred from the old cultures into 30 mL fresh medium in 100 mL Erlenmeyer flask. For the transgenic lines, 1.5 mL cells were transferred from the old cultures into 30 mL fresh medium in 100 mL Erlenmeyer flask. BY-2 Tuβ6 cell line was supplemented with 50 mg/L kanamycin and GF-11 line was supplemented with 30 mg/L hygromycin. Both transgenic and non-transgenic lines were cultured on KS260 horizontal shaker (IKA Labortechnik, Staufen, Germany) at 150 rpm in a dark room at 25 ℃.

2.2 Rice Cultivation

2.2.1 Rice Cultivar

The *Oryza sativa* variety used in this research was a japonica variety named Dongjin, which was developed by the Rural Development Administration of Korea (So-Hyeon 2013). The Dongjin seeds used in this research were harvested in 2010 and 2014, and they were stored in 4 ℃ in darkness. Dongjin variety could germinate and grow root in less than ten days, suitable for seedling rice root experiments.

2.2.2 Darkness cultivation for root growth

Rice seedling root was required in this research, therefore Dongjin seeds were cultivated in dark to stimulate its root growth. Seed husk was removed by a hand grinder firstly, then the naked seeds were placed on a plastic mesh embryo facing down and have been transferred into a plastic cubic container with water. The cubic container would be put into a total dark environment with 27 ℃. The mesh would hold the seeds floating on the water surface, and the root could grow freely downward. This germination and root growth stage would last for 5 days.

2.2.3 Cultivation in incubator

To investigate the toxicity of CPPo to rice, rice seedling's root growth condition has been checked after it treated with CPPo. After the germination as described in 2.2.2 and CPPo treatment, rice seedlings were transfer to a cylinder container and continued to be cultivated in a incubator. The temperature in the incubator is constant 27℃ and the day/night ratio is 16h/8h. Rice seedling will be cultivated five days in this incubator.

2.3 CPPo library

The whole CPPo library was synthesised and provided by Porf. Dr. Ute Schepers in Institute of Toxicology and Genetics (ITG), KIT. The members of this library shares a common chemical structure (shown in Figure 2.1) of a tetrameric peptoids with four side chains on each nitrogen atom in the back bone. It also has a rhodanmine to the end of peptoid backbone as reporter molecule, so the movement and localisation could be tracked under microscope with 588 nm florescent light.

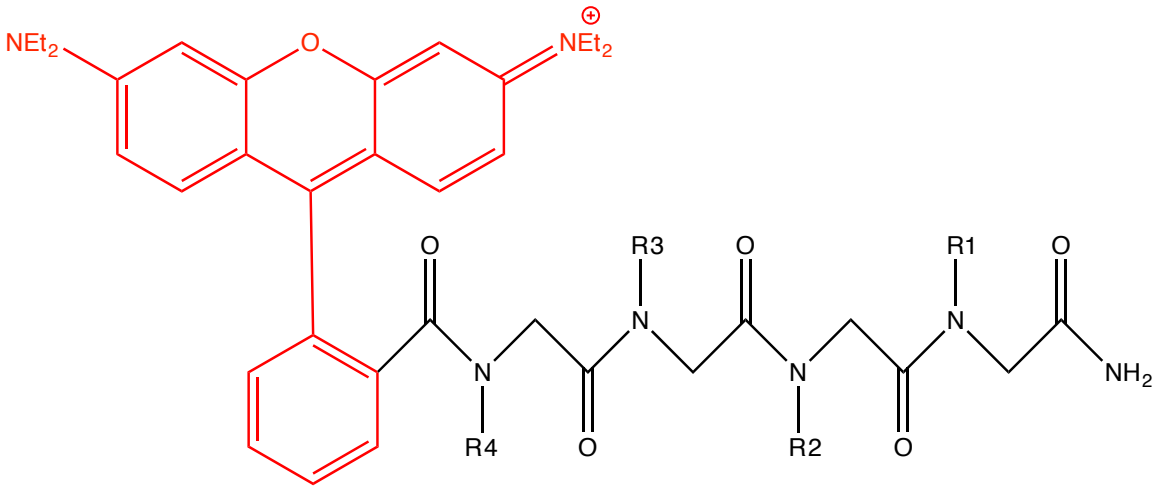


Figure 2.1The structure of individual CPPo in the library

The general structure of individual CPPo is a tetrameric structure peptoids. Unlike peptide, the R residue in peptoid is linked to nitrogen instead of carbon atom. As a result it has more resistance to enzymes that can cleve peptide bond. In this library, the side chains (R residue) has four choices.The red part of is rhodamine residue.

There are four nitrogen atom available for four different side chains(shown in Figure 2.2) in the backbone, therefore theoretically the whole library should have 256 different peptoids. However in this study's case, 239 peptoids were available in the library for cellular level research, and 160 peptoids for organ level research.

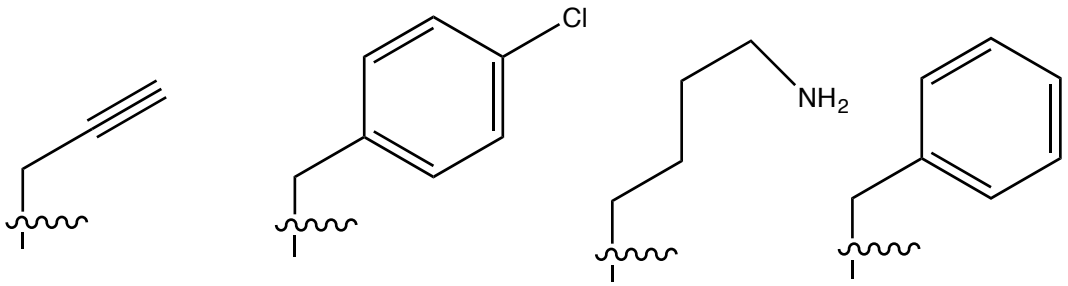


Figure 2.2 The structure of side chains of CPPo in the library
The affinity of these side chains are different, and it could influence the penetration ability of the CPPo. The third side chain is hydrophilic, and the rest of them are lipophilic.

The CPPo library have been stored in darkness in 4℃ refrigerator or cold room. The storage concentration is 20 μM.

2.4 Cell phenotying technique and blocker treatment

2.4.1 Cell mortality

The cell mortality was tested using Evans blue dye which was named after an American scientist Herbert McLean Evans. When the cell membrane was intact, Evans blue would not be able to penetrate inside cells, so living cell would remain transparent. Otherwise Evans blue would penetrate into cells, thus the dead or dying cells would be blue under microscope observation.

For the dyeing process, 1 mL BY-2 suspension cells would be put in the self-made washing chamber (described in 2.5.3), drain the medium with tissue paper. Then cells would be transferred into 2.5% (w/v) Evans blue dye dissolved in fresh medium for 3 minutes (Gaff and Okong'O-Ogola, 1971) . Drained the liquid dye with tissue paper and rinsed the cells with fresh MS medium for three times. Then the cells would be ready to be inspected under microscope.

Cell mortality was calculated by the following formula：

Cell mortality = (Number of dead cell/ Total cell number)×100%

In this research, the total cell number is 500.

2.4.2 CPPo and cytoskeleton co-localisation experiment

For the microtubule co-localisation experiment Tuβ6 cell line was used. 995 µL three days old Tuβ6 cells were transferred from erlenmeyer flask into 24 well plate, 5 µL 20 µM stock CPPo were added in. CPPo treated Tuβ6 cells were put on a 200 rpm horizontal shaker shaking at 27 °C. After the two hours incubation, cells were rinsed with fresh sterile culture medium and observed immediately.

For the actin co-localisation experiment GF11 cell line was used. Other treatments are as same as microtubule co-localisation experiment.

2.4.3 Actin inhibitor latrunculin B treatment

For the actin inhibitor experiment, latrunculin B (Spector et al., 1983) (Sigma-Aldrich) was used. Latrunculin B was stocked in DMSO with concentration 1 mM, and then diluted with fresh MS medium to a final concentration of 500 nM. BY-2 cells were incubated for 12 hours (Maisch et al., 2009) in 24 well plates on a horizontal shaker. Then 2 µM CPPo was adding to the latrunculin B treated cells and cells were continued to be incubated on horizontal shaker with 200 rpm for two more hours at 27 °C, then rinsed with fresh sterile culture medium and observed immediately.

2.4.4 Microtubule inhibitor oryzalin

As for another member of cell skeleton in plant cell, microtubule also play an important role in plant cell metabolism. To investigate the function of microtubule in CPPo distribution inside BY-2 cells, microtubules were eliminated by oryzalin Chem Service, West Chester, PA, USA) (Morejohn et al., 1987) . Oryzalin was diluted in DMSO to 10 mM as stock solution and store in 4℃. Before experiments, oryzalin was diluted with fresh MS medium to a final concentration of 5 µM. BY-2 cells were then incubated for 12 hours with the oryzalin-containing medium, which is sufficient to remove microtubules (Schwarzerova et al., 2006). This procedure was done in 24 well plates on a 200 rpm rotating horizontal shaker. After pretreatment of cell with oryzalin, 2 µM CPPo were added, and cells were

incubated for two more hours as described above. Subsequently, the cells were washed using fresh sterile culture medium and observed immediately.

2.4.5 Myosin inhibitor BDM

For the myosin inhibitor treatment, 2,3-butanedione monoxime (BDM, Sigma-Aldrich) (Tominaga et al., 2000) was used. BDM was stocked at a final concentration of 5 mM as an aqueous stock, before added to BY-2 cells, BDM was diluted to 10 μM or 30 μM (Holweg et al., 2003) and then added to BY-2 cells.The cells were incubated over night at 27 °C for 12 hours under continuous shaking in a 24 well plate on a 200 rpm horizontal shaker. Subsequently, 2 μM CPPo was added to the cells and cells were incubated for two more hours on the shaker, then the cells were rinsed as described before and observed immediately under microscope.

2.4.6 Visualization of mitochondria

For mitochondria visualization process, 100 nM freshly prepared Mito Tracker Green were applied to BY-2 cells. Mito Tracker Green were stocked of 100 μM in dimethyl sulfoxide (DMSO) in 4 °C. The washing step could be escaped due to Mito Tracker Green could only be fluorescent after accumulate in mitochondrial lipid environment (Agnello et al., 2008).

2.5 CPPo treatment

2.5.1 Manufacture of self-made washing chamber

In many experiments tobacco BY-2 cells need to be washed off different blockers, CPPos or other dyes. However no market sold instrument is available, thus a self-made washing chamber is necessary. In this section, an instruction of a simple way to make a cell washing chamber with filter mesh and eppendorf tubes is shown.

Material required: 1.5 mL eppendorf tubes, filter meshes (Franz Eckert PA-11/6).
Equipments required: scissors and heating device (a magnetic stirrer with heating ability was used in this research).

Step one: remove the bottom part of eppendorf tube with scissor, smooth the edge.

Step two: melt the newly cut edge of eppendorf tube with magnetic mixer, temperature should between 150℃ to 200℃.

Step three: put the eppendorf tube on the filter mesh while the edge still melted. The melted plastic will perform as glue, and seal filter mesh and eppendorf tube together.

Step four: cut the washing chamber down from filter mesh, wait till it totally cool down and test it whether leak or not.

2.5.2 BY-2 cells

In order to investigate the penetration ability, distribution pattern and penetration mechanisms of CPPo into plant cell, BY-2 suspension cell was used as a model system. As described in 2.1.2, BY-2 suspension cells were subcultured in a weekly cycle, to make sure that the cells are robust, healthy and in a relatively vigorously division phase, three days old suspension cell were used in experiments.

Transfer 995 µL suspension cells from erlenmeyer flask into a well in 24 well plates. 5 µL CPPo were added into cells, and the working concentration was 1 µM. Cells were shaken on horizontal shaker for two hours with speed 200 rpm. This rotation speed is faster than the speed used when subculture due to shorter diameter of the well of 24 well plate than erlenmeyer flask. Faster speed could keep cells shaking inside of the well instead of bouncing against the wall.

Cells in 24 well plate would be shake for two hours and then be washed in self-made washing chamber with fresh MS medium that used to subculture BY-2 cells for three times to get rid of excessive CPPo. Then BY-2 cells could be observed under microscope.

2.5.3 Rice seedlings

In order to research the behaviour of CPPo in real plants, they have been applied to rice seedlings. As described in 2.2, Dongjin seed would be cultivated in dark environment for five days to grow root. After this phase, Dongjin seedling were incubated in 2 mL erlenmeyer tube containing 1 μM CPPo dissolved in distill water.

After two hours incubation, rice seedling was taken out, and washed with distill water for three times. In this process, washing step would use running water to prevent root suck in fresh water and have unpredictable effect on the distribution of CPPo in the rice root tip.

Following the washing step, the rice tip was cut down and then observed under microscope.

2.5.4 Rice health

In order to find out whether the rice would still grow normally after been treated with CPPo, rice seedling were treated with three CPPo candidates represent each distribution in suspension cells by the method described in 2.5.3. Then the length of shoots and roots were measured. After this process, rice seedlings were incubated in a incubator as described in 2.2.3. On the fifth day of incubation in incubator, length of roots and shoots were measured again.

2.6 Microscopy

2.6.1 Microscopical setup

For cell phenotyping analysis, a Zeiss microscope (AxioImager Z.1) was used. This microscope is equipped with an ApoTome microscope slider for optical section purpose, and the camera on this microscope is a cooled digital CCD camera (AxioCam MRm). For

observation of immortality of BY-2 cells, 20× objective lens (Plan-Apochromat 20x/0.75) and differential interference contrast (DIC) filter was used (Zeiss, Jena, Germany).

For CPPo classification, distribution experiments, an AxioObserver Z1 (Zeiss, Jena, Germany) inverted microscope equipped with a laser dual spinning disk scan head from Yokogawa (Yokogawa CSU-X1 Spinning Disk Unit, Yokogawa Electric Corporation, Tokyo, Japan), a cooled digital CCD camera (AxioCam MRm; Zeiss) and two laser lines (488 and 561 nm, Zeiss, Jena, Germany) was used. This confocal microscope is equipped with a spinning disc scan head for optical section purpose. Image was take with a 64× oil objective lens.

2.6.2 Image Analysis

For Analysis images acquired by AxioObserver Z1 microscope , software Zen 2 (blue edition, acquired from Zeiss company website freely) and Lightroom (Adobe) was used.

For the quality experiments, CPPo signal was enhanced and noise in the background was reduced. These two steps were performed with Zen 2.

3 Results

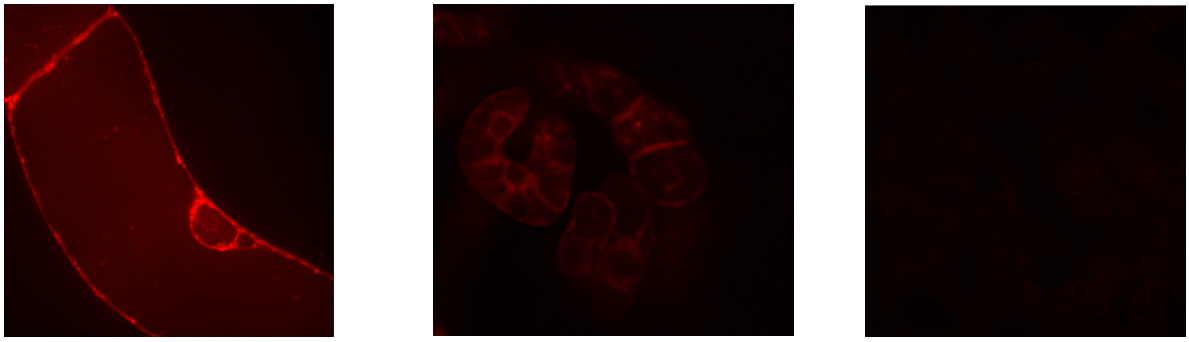
3.1 The Behaviours of CPPo Library in BY-2 Cells

Although CPPs and CPPos are discussed as one category in most of time, but each and everyone of them has different chemical and biological traits. Therefore when a new CPP or CPPo has been discovered or synthesised, its behaviour on different biological level should be examined all over again. In this research, the behaviours of CPPo library on cell level have been tested first.

3.1.1 The Classification of CPPo library by the entry efficiency

To test the penetration ability of CPPos in the library, 1 mL BY-2 cells were incubated with each CPPo in the library in 24-well plate on a shaker for 2 hours. Immediately after the incubation phase, excess CPPo was removed and the cells were observed using spinning disc microscope with 588 nm laser. Due to the concentration of CPPo and penetration time were the same, the intensity of florescent light of CPPo inside BY-2 cells could be one index of the CPPo entry efficiency.

By the observation of the signal intensity, CPPos have been arbitrarily categorised into three class. Class one: strong signal, meaning this CPPo has good penetration ability and high penetration rate, suitable for further studies and possible application usage. Class two: medium signal, meaning this CPPo has moderate penetration ability and moderate penetration rate, suitable for some scientific studies and some particular applications such as low concentration drug delivery. Class three: weak signal, meaning this CPPo has weak penetration ability and low penetration rate, is not suitable for scientific researches and has limited usage in industry field. The typical pictures of class one, two and three CPPo are shown in Figure 3.1. These pictures were taken by AxioObserver Z1, 64× oil objective lens and 561 nm laser with 100% intensity.



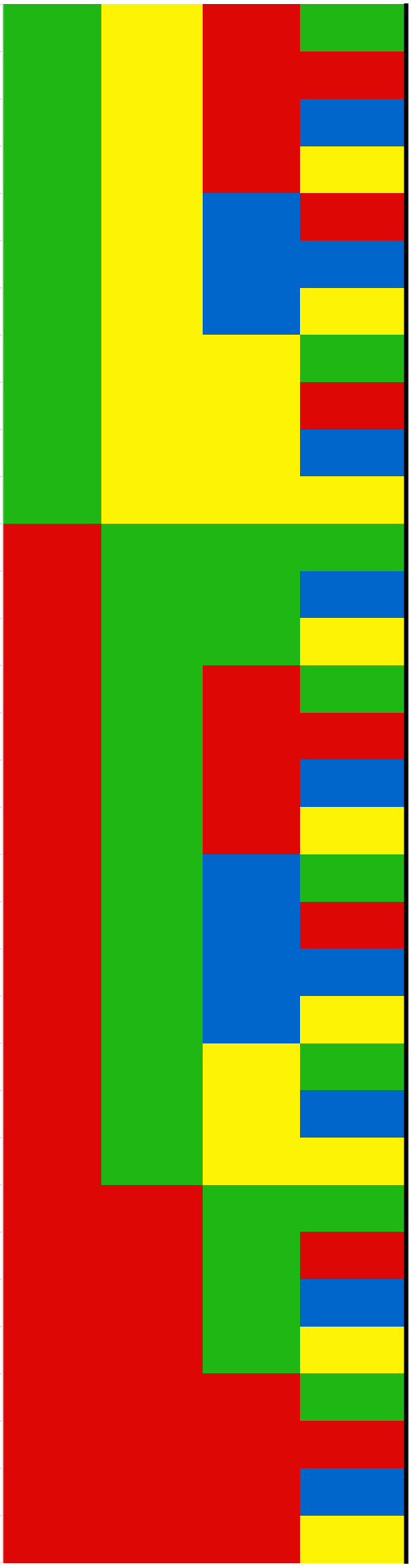
a b c

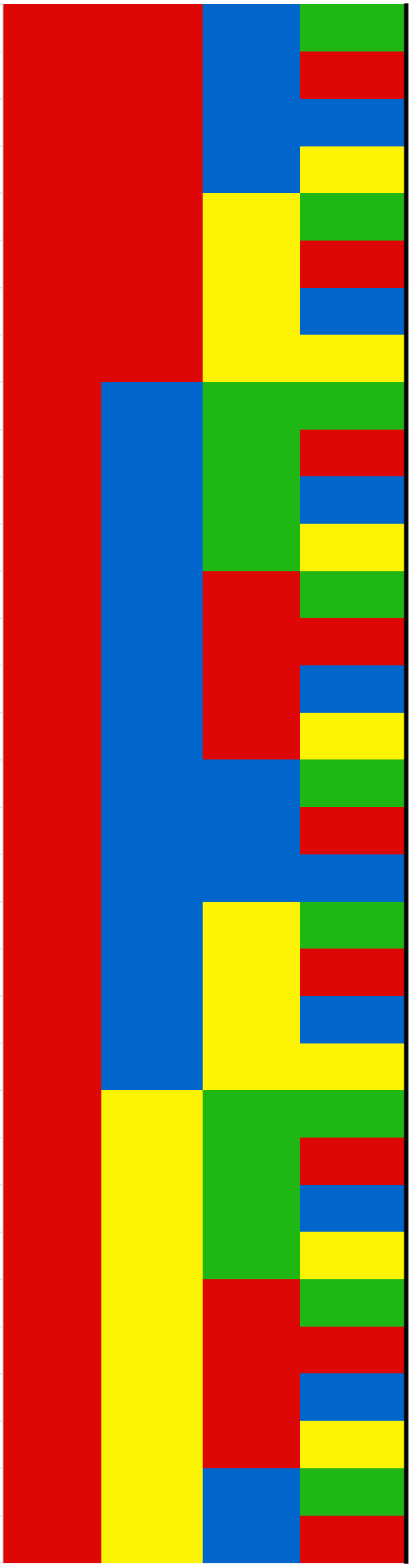
Figure 3.1 Original pictures of CPPo in BY-2 cells.
a is a picture of peptoid 38 which is a class one peptoid, b is a picture of peptoid 8 which is a class two peptoid, c is a picture of peptoid 25 which is a class three peptoid.

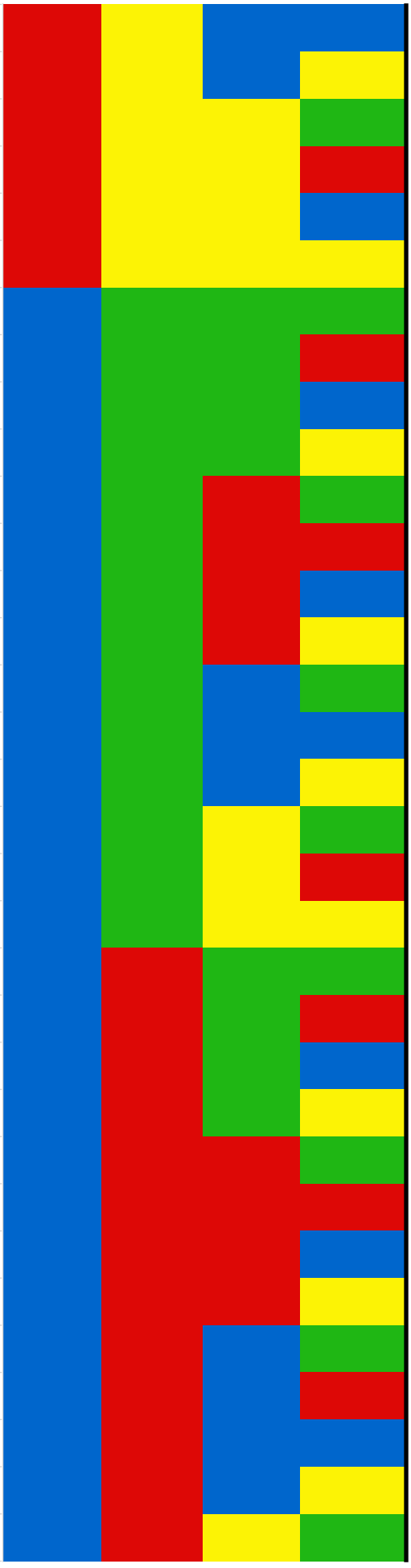
The classification result is shown in table 3.1. Number in classification with number code column shows the class where this CPPo belongs to.

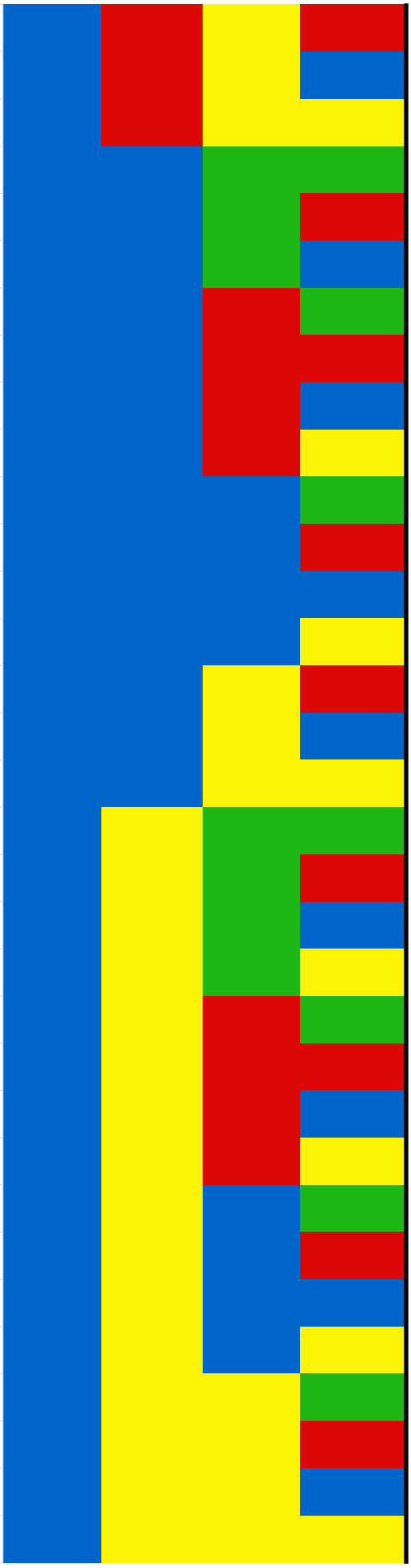
Peptoid	Molecular weight	Classification with number code	Sidechain with number code	Sidechain with color code
P1	822,49	3	1 1 1 1	
P2	908,48	3	1 1 1 2	
P3	855,55	3	1 1 1 3	
P4	874,52	3	1 1 1 4	
P5	908,48	1	1 1 2 1	
P6	994,347	3	1 1 2 2	
P7	941,53	3	1 1 2 3	
P8	960,51	2	1 1 2 4	
P9	855,54	3	1 1 3 1	
P10	941,53	3	1 1 3 2	
P11	888,59	2	1 1 3 3	
P13	874,52	3	1 1 4 1	
P14	960,51	2	1 1 4 2	
P15	907,57	3	1 1 4 3	
P17	908,48	3	1 2 1 1	
P18	994,47	3	1 2 1 2	

P19	941,53	2	1 2 1 3	
P20	960,51	3	1 2 1 4	
P22	1080,46	2	1 2 2 2	
P23	1029,52	3	1 2 2 3	
P24	1046,5	3	1 2 2 4	
P25	941,53	3	1 2 3 1	
P26	1027,52	2	1 2 3 2	
P27	974,58	3	1 2 3 3	
P28	993,56	3	1 2 3 4	
P29	960,51	3	1 2 4 1	
P30	1046,5	3	1 2 4 2	
P31	993,56	3	1 2 4 3	
P32	1012,54	3	1 2 4 4	
P33	855,54	3	1 3 1 1	
P34	941,53	3	1 3 1 2	
P35	888,59	3	1 3 1 3	
P36	907,57	3	1 3 1 4	
P37	941,53	1	1 3 2 1	
P38	1027,52	1	1 3 2 2	
P39	974,58	3	1 3 2 3	
P40	993,56	3	1 3 2 4	
P41	888,59	3	1 3 3 1	
P42	974,58	3	1 3 3 2	
P43	921,64	3	1 3 3 3	
P44	940,62	3	1 3 3 4	
P45	907,57	3	1 3 4 1	
P46	993,56	3	1 3 4 2	
P47	940,62	2	1 3 4 3	
P48	959.6	3	1 3 4 4	
P49	874,52	3	1 4 1 1	
P50	960,51	3	1 4 1 2	
P51	907,57	3	1 4 1 3	
P52	926,55	1	1 4 1 4	

P53	960,51	3	1 4 2 1	
P54	1046,5	1	1 4 2 2	
P55	993,56	3	1 4 2 3	
P56	1012,54	3	1 4 2 4	
P58	993,56	3	1 4 3 2	
P59	940,62	3	1 4 3 3	
P60	959,6	3	1 4 3 4	
P61	926,55	3	1 4 4 1	
P62	1012,54	3	1 4 4 2	
P63	959,6	3	1 4 4 3	
P64	978,58	3	1 4 4 4	
P65	908,48	3	2 1 1 1	
P67	941,53	3	2 1 1 3	
P68	960,51	3	2 1 1 4	
P69	994,51	3	2 1 2 1	
P70	1080,46	3	2 1 2 2	
P71	1027,52	3	2 1 2 3	
P72	1046,5	1	2 1 2 4	
P73	941,53	1	2 1 3 1	
P74	1027,52	3	2 1 3 2	
P75	974,58	3	2 1 3 3	
P76	993,56	2	2 1 3 4	
P77	960,51	1	2 1 4 1	
P79	993,56	3	2 1 4 3	
P80	1012,54	1	2 1 4 4	
P81	994,47	1	2 2 1 1	
P82	1080,46	3	2 2 1 2	
P83	1027,52	2	2 2 1 3	
P84	1046,5	2	2 2 1 4	
P85	1080,46	3	2 2 2 1	
P86	1166,45	1	2 2 2 2	
P87	1113,51	2	2 2 2 3	
P88	1132,49	1	2 2 2 4	

P89	1027,52	2	2 2 3 1	
P90	1113,51	2	2 2 3 2	
P91	1060,57	3	2 2 3 3	
P92	1079,55	1	2 2 3 4	
P93	1046,5	1	2 2 4 1	
P94	1132,49	1	2 2 4 2	
P95	1079,55	3	2 2 4 3	
P96	1098,53	3	2 2 4 4	
P97	941,53	2	2 3 1 1	
P98	1027,52	3	2 3 1 2	
P99	974,58	3	2 3 1 3	
P100	993,56	3	2 3 1 4	
P101	1027,52	3	2 3 2 1	
P102	1113,51	1	2 3 2 2	
P103	1060,57	2	2 3 2 3	
P104	1079,55	2	2 3 2 4	
P105	974,58	3	2 3 3 1	
P106	1060,57	3	2 3 3 2	
P107	1007,63	3	2 3 3 3	
P109	993,56	3	2 3 4 1	
P110	1079,55	3	2 3 4 2	
P111	1026,61	3	2 3 4 3	
P112	1045,59	3	2 3 4 4	
P113	960,51	3	2 4 1 1	
P114	1046,5	3	2 4 1 2	
P115	993,56	3	2 4 1 3	
P116	1012,54	3	2 4 1 4	
P117	1046,5	3	2 4 2 1	
P118	1132,49	1	2 4 2 2	
P119	1079,55	3	2 4 2 3	
P120	1098,53	2	2 4 2 4	
P121	993,56	3	2 4 3 1	
P122	1079,55	1	2 4 3 2	

P123	1026,61	3	2 4 3 3	
P124	1045,59	2	2 4 3 4	
P125	1012,54	3	2 4 4 1	
P126	1098,53	1	2 4 4 2	
P127	1045,59	3	2 4 4 3	
P128	1064,57	1	2 4 4 4	
P129	855,54	3	3 1 1 1	
P130	941,53	3	3 1 1 2	
P131	888,59	3	3 1 1 3	
P132	907,57	3	3 1 1 4	
P133	941,53	2	3 1 2 1	
P134	1027.52	1	3 1 2 2	
P135	974,58	2	3 1 2 3	
P136	993,56	3	3 1 2 4	
P137	888,59	2	3 1 3 1	
P139	921,64	3	3 1 3 3	
P140	940,62	3	3 1 3 4	
P141	907,57	3	3 1 4 1	
P142	993,56	3	3 1 4 2	
P144	959,6	3	3 1 4 4	
P145	941,53	1	3 2 1 1	
P146	1027,52	3	3 2 1 2	
P147	974,58	3	3 2 1 3	
P148	993,56	3	3 2 1 4	
P149	1027,52	2	3 2 2 1	
P150	1113,51	3	3 2 2 2	
P151	1060,57	3	3 2 2 3	
P152	1079,55	2	3 2 2 4	
P153	974,58	3	3 2 3 1	
P154	1060,57	3	3 2 3 2	
P155	1007,63	1	3 2 3 3	
P156	1026,61	3	3 2 3 4	
P157	993,56	2	3 2 4 1	

P158	1079,55	2	3 2 4 2	
P159	1026,61	2	3 2 4 3	
P160	1045,59	3	3 2 4 4	
P161	888,59	2	3 3 1 1	
P162	9974,58	3	3 3 1 2	
P163	921,64	3	3 3 1 3	
P165	974,58	1	3 3 2 1	
P166	1060,57	3	3 3 2 2	
P167	1007,63	3	3 3 2 3	
P168	1026,61	3	3 3 2 4	
P169	921,64	3	3 3 3 1	
P170	1007,63	3	3 3 3 2	
P171	954,69	3	3 3 3 3	
P172	973,667	2	3 3 3 4	
P174	1026,61	3	3 3 4 2	
P175	973,67	3	3 3 4 3	
P176	992,65	2	3 3 4 4	
P177	907,57	3	3 4 1 1	
P178	993,56	2	3 4 1 2	
P179	940,62	3	3 4 1 3	
P180	959,6	1	3 4 1 4	
P181	993.56	1	3 4 2 1	
P182	1079,55	1	3 4 2 2	
P183	1026,61	3	3 4 2 3	
P184	1045,59	3	3 4 2 4	
P185	940,62	1	3 4 3 1	
P186	1026,61	1	3 4 3 2	
P187	973,67	2	3 4 3 3	
P188	992,65	2	3 4 3 4	
P189	959,6	2	3 4 4 1	
P190	1045,59	1	3 4 4 2	
P191	992,65	2	3 4 4 3	
P192	1011,63	3	3 4 4 4	

P193	874,52	1	4 1 1 1	
P194	960,51	1	4 1 1 2	
P195	907,57	2	4 1 1 3	
P196	926,55	1	4 1 1 4	
P197	960,51	1	4 1 2 1	
P198	1046,5	1	4 1 2 2	
P199	993,56	2	4 1 2 3	
P200	1012,54	2	4 1 2 4	
P201	907,57	1	4 1 3 1	
P202	993,56	2	4 1 3 2	
P203	940,62	3	4 1 3 3	
P204	959,6	1	4 1 3 4	
P205	926,55	2	4 1 4 1	
P206	1012,54	3	4 1 4 2	
P207	959,6	3	4 1 4 3	
P208	978,58	1	4 1 4 4	
P209	960,51	2	4 2 1 1	
P210	1046,5	2	4 2 1 2	
P211	993,56	3	4 2 1 3	
P212	1012,54	2	4 2 1 4	
P213	1046,5	3	4 2 2 1	
P214	1132,49	2	4 2 2 2	
P215	1079,55	3	4 2 2 3	
P216	1098,53	2	4 2 2 4	
P217	993,56	3	4 2 3 1	
P218	1079,55	3	4 2 3 2	
P219	1026,61	3	4 2 3 3	
P220	1045,59	2	4 2 3 4	
P221	1012,54	2	4 2 4 1	
P222	1098,53	2	4 2 4 2	
P223	1045,59	3	4 2 4 3	
P225	907,57	3	4 3 1 1	
P226	993,56	3	4 3 1 2	

P227	940,62	3	4 3 1 3	
P228	959,6	2	4 3 1 4	
P229	993,56	3	4 3 2 1	
P230	1079,55	3	4 3 2 2	
P232	1045,59	3	4 3 2 4	
P233	940,62	3	4 3 3 1	
P234	1026,61	3	4 3 3 2	
P236	992,65	3	4 3 3 4	
P237	959360	3	4 3 4 1	
P238	1045,59	3	4 3 4 2	
P239	992,65	3	4 3 4 3	
P240	1011,63	3	4 3 4 4	
P241	926,55	3	4 4 1 1	
P242	1012,54	3	4 4 1 2	
P243	959,6	3	4 4 1 3	
P244	978,58	2	4 4 1 4	
P245	1012,54	3	4 4 2 1	
P246	1098,53	3	4 4 2 2	
P247	1045,59	3	4 4 2 3	
P248	1064,57	3	4 4 2 4	
P249	959,6	3	4 4 3 1	
P250	1045,59	3	4 4 3 2	
P251	992,65	3	4 4 3 3	
P252	1011,63	3	4 4 3 4	
P253	978,58	3	4 4 4 1	
P255	1011,63	3	4 4 4 3	
P256	1030,61	3	4 4 4 4	

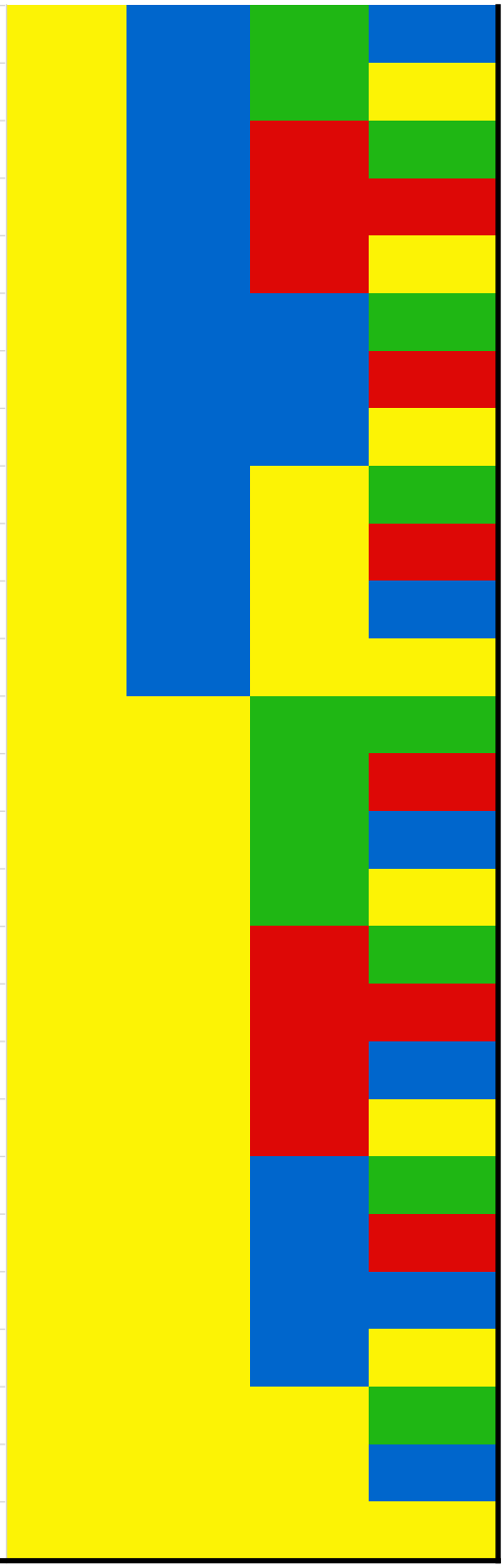


Table 3.1 The classification of CPPo based on its penetration efficiency (arbitrary)
The first column stands for each individual CPPo in the library based on its serial number. The second column stands for each molecular weight. The third column stands for its classification base on penetration efficiency . The fourth and fifth column stand for two methods of coding CPPo shown in figure 3.4

3.1.2 The CPPo library do not harm BY-2 cells

There is one trait of CPP or CPPo is pivotal to the future engineering utilization of these chemicals: whether this particular CPP or CPPo will effluence organism in some way. If it does, it could be used as inhibitor, tracker or something else according to the specific ability. In this case, this CPP or CPPo could perform as a drug by itself or it could carry a cargo molecule to have a dual effects at the same time. If not, it could be only used as a vehicle to transport cargo molecule into target area. However it will not harm the organism in any form, so it will become an ideal molecule transporter.

To test whether the CPPos in this library will be harmful or not, they have been applied to BY-2 cell (*Nicotiana tabacum* L. cv. Bright Yellow 2) and the cell mortality had been examined. Figure 3.2 tells that BY-2 suspension cells could survive CPPo treatment very well.

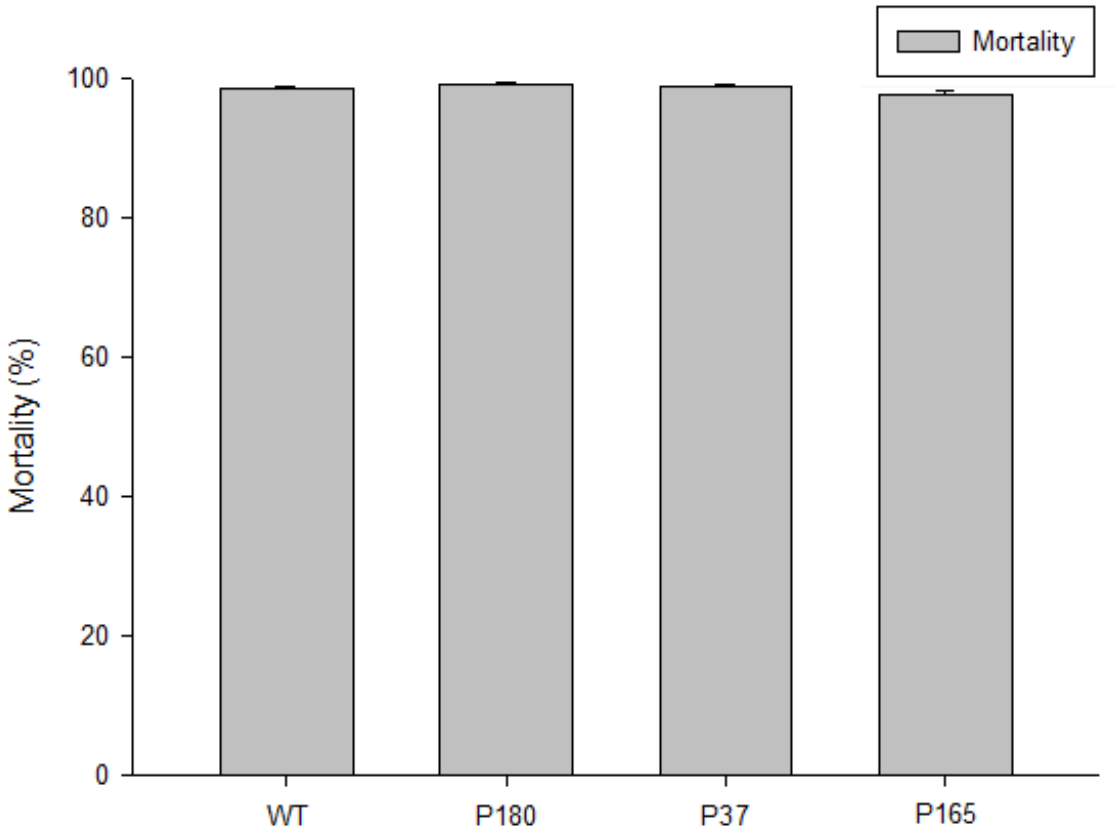
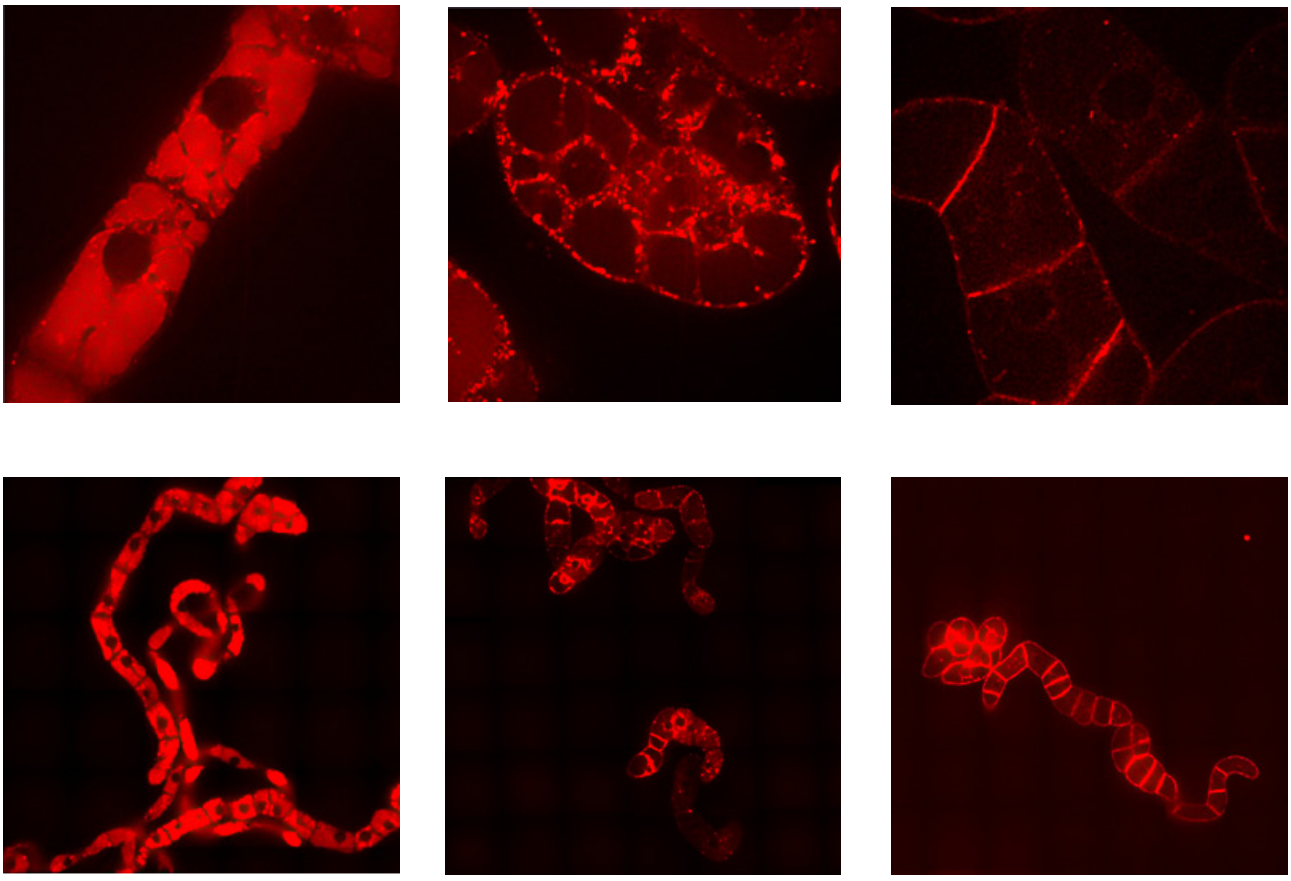


Figure 3.2 Mortality of CPPo treated cells

WT is the control group while the rest three are the BY-2 cells treated by CPPo respectively. It is clear that with the CPPo treatment, 3 days old BY-2 cells could remain at a high level of mortality, all above 95%.

3.1.3 The distribution pattern of CPPo library inside BY-2 cell

During the 2 hours incubation time, CPPo in the library penetrated into BY-2 cells with different efficiency. Although most of them have been classified into 3rd category, but with the help of software ZEN, the intensity of CPPo signal could be increased meanwhile the background could be darkened. By this method, all the CPPo became visible and three different distribution pattern were discovered.



a b c

Figure 3.3 Three patterns of CPPo distribution in BY-2 cells
column a are pictures of P180 and vacuole pattern, column b are pictures of P156 and dot pattern, column c are pictures of P116 and membrane pattern

Nearly all of the CPPo in the library have been tested, and some of the distribution pattern pictures are displayed in Figure 3.3. In column a, vacuole pattern was shown. In this pattern, CPPo spreads evenly like cloud inside the cell except the nucleus. In column b,

dot pattern was shown. In this pattern, CPPo formed small dots and distributed in cytoplasm, but not in nucleus and vacuole. In column c, membrane was shown. In this pattern, CPPo located mostly on the cell membrane.

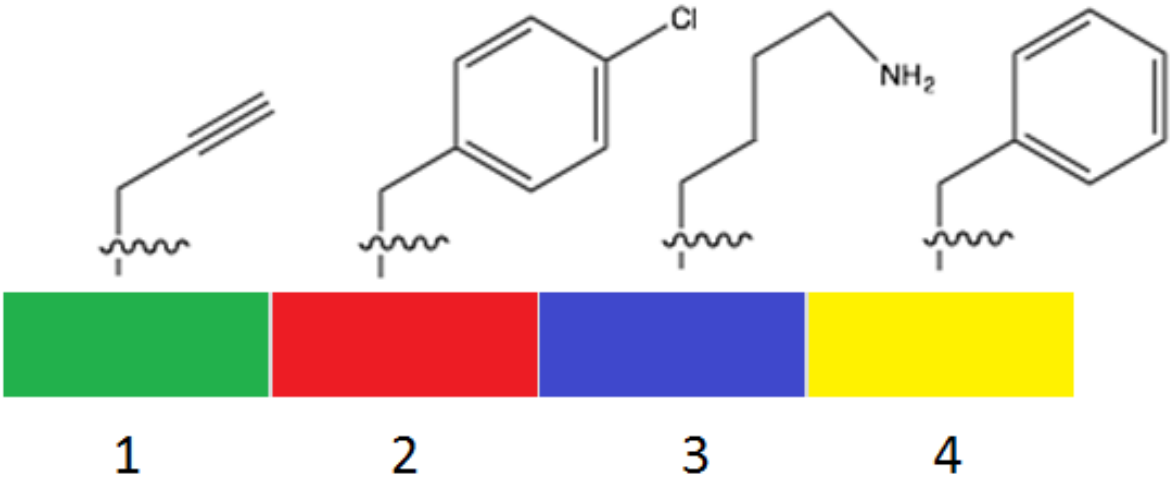


Figure 3.4 The colour and number code of side chain structures

A table (Table 3.2) has been made according to the distribution pattern of each CPPo in BY-2 cells and the chemical structure of the CPPo. In chapter 2.3, the general structure of CPPo has been displayed. There are four R residues (Figure 2.1), due to the asymmetric structure, these four R residues are chemically different and are marked as R1, R2, R3 and R4. And for the four kinds of side chain chemical structures, a number and colour code was made (Figure 3.4).

Peptoid	Molecular weight	Classification with letter code	Classification with color code	Sidechain with number code	Sidechain with color code
P1	822,49	A		1 1 1 1	
P2	908,48	A		1 1 1 2	
P3	855,55	A		1 1 1 3	
P4	874,52	B		1 1 1 4	
P5	908,48	B		1 1 2 1	
P6	994,347	Ca		1 1 2 2	
P7	941,53	A		1 1 2 3	
P8	960,51	Ab		1 1 2 4	
P9	855,54	Ab		1 1 3 1	
P10	941,53	C		1 1 3 2	
P11	888,59	B		1 1 3 3	
P13	874,52	Ab		1 1 4 1	

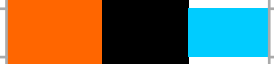
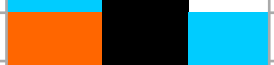
Peptoid	Molecular weight	Classification with letter code	Classification with color code	Sidechain with number code	Sidechain with color code
P14	960,51	Cab		1 1 4 2	
P15	907,57	B		1 1 4 3	
P17	908,48	B		1 2 1 1	
P18	994,47	B		1 2 1 2	
P19	941,53	Cb		1 2 1 3	
P20	960,51	Ba		1 2 1 4	
P22	1080,46	B		1 2 2 2	
P23	1029,52	B		1 2 2 3	
P24	1046,5	C		1 2 2 4	
P25	941,53	B		1 2 3 1	
P26	1027,52	C		1 2 3 2	
P27	974,58	B		1 2 3 3	
P28	993,56	Cb		1 2 3 4	
P29	960,51	Ca		1 2 4 1	
P30	1046,5	C		1 2 4 2	
P31	993,56	C		1 2 4 3	
P32	1012,54	B		1 2 4 4	
P33	855,54	Ba		1 3 1 1	
P34	941,53	Ab		1 3 1 2	
P35	888,59	Ab		1 3 1 3	
P36	907,57	Ba		1 3 1 4	
P37	941,53	Ba		1 3 2 1	
P38	1027,52	Cb		1 3 2 2	
P39	974,58	Cab		1 3 2 3	
P40	993,56	Bac		1 3 2 4	
P41	888,59	Ba		1 3 3 1	
P42	974,58	Ba		1 3 3 2	
P43	921,64	Ab		1 3 3 3	
P44	940,62	B		1 3 3 4	
P45	907,57	Ab		1 3 4 1	
P46	993,56	Ca		1 3 4 2	
P47	940,62	B		1 3 4 3	
P48	959.6	Ab		1 3 4 4	
P49	874,52	B		1 4 1 1	
P50	960,51	Bac		1 4 1 2	
P51	907,57	Cb		1 4 1 3	
P52	926,55	A		1 4 1 4	

Peptoid	Molecular weight	Classification with letter code	Classification with color code	Sidechain with number code	Sidechain with color code
P53	960,51	B		1 4 2 1	
P54	1046,5	Bac		1 4 2 2	
P55	993,56	B		1 4 2 3	
P56	1012,54	Ab		1 4 2 4	
P58	993,56	A		1 4 3 2	
P59	940,62	B		1 4 3 3	
P60	959,6	Ab		1 4 3 4	
P61	926,55	Ab		1 4 4 1	
P62	1012,54	Ab		1 4 4 2	
P63	959,6	B		1 4 4 3	
P64	978,58	B		1 4 4 4	
P65	908,48	C		2 1 1 1	
P67	941,53	B		2 1 1 3	
P68	960,51	Abc		2 1 1 4	
P69	994,51	Ba		2 1 2 1	
P70	1080,46	Ba		2 1 2 2	
P71	1027,52	B		2 1 2 3	
P72	1046,5	Ab		2 1 2 4	
P73	941,53	Cab		2 1 3 1	
P74	1027,52	Ca		2 1 3 2	
P75	974,58	Cb		2 1 3 3	
P76	993,56	Cab		2 1 3 4	
P77	960,51	Ba		2 1 4 1	
P79	993,56	Cab		2 1 4 3	
P80	1012,54	Cab		2 1 4 4	
P81	994,47	Cab		2 2 1 1	
P82	1080,46	Cb		2 2 1 2	
P83	1027,52	Ca		2 2 1 3	
P84	1046,5	Cb		2 2 1 4	
P85	1080,46	B		2 2 2 1	
P86	1166,45	B		2 2 2 2	
P87	1113,51	Cb		2 2 2 3	
P88	1132,49	Cb		2 2 2 4	
P89	1027,52	Cb		2 2 3 1	
P90	1113,51	Cb		2 2 3 2	
P91	1060,57	Ab		2 2 3 3	
P92	1079,55	Cb		2 2 3 4	

Peptoid	Molecular weight	Classification with letter code	Classification with color code	Sidechain with number code	Sidechain with color code
P93	1046,5	Bc		2 2 4 1	
P94	1132,49	B		2 2 4 2	
P95	1079,55	B		2 2 4 3	
P96	1098,53	Ba		2 2 4 4	
P97	941,53	Ba		2 3 1 1	
P98	1027,52	Cab		2 3 1 2	
P99	974,58	Cab		2 3 1 3	
P100	993,56	B		2 3 1 4	
P101	1027,52	Cb		2 3 2 1	
P102	1113,51	Cb		2 3 2 2	
P103	1060,57	C		2 3 2 3	
P104	1079,55	Cb		2 3 2 4	
P105	974,58	B		2 3 3 1	
P106	1060,57	Ca		2 3 3 2	
P107	1007,63	Ca		2 3 3 3	
P109	993,56	Ca		2 3 4 1	
P110	1079,55	Cab		2 3 4 2	
P111	1026,61	Ab		2 3 4 3	
P112	1045,59	Cb		2 3 4 4	
P113	960,51	A		2 4 1 1	
P114	1046,5	Ca		2 4 1 2	
P115	993,56	Cab		2 4 1 3	
P116	1012,54	Ca		2 4 1 4	
P117	1046,5	A		2 4 2 1	
P118	1132,49	B		2 4 2 2	
P119	1079,55	Ab		2 4 2 3	
P120	1098,53	Bc		2 4 2 4	
P121	993,56	A		2 4 3 1	
P122	1079,55	Cb		2 4 3 2	
P123	1026,61	Cb		2 4 3 3	
P124	1045,59	Ca		2 4 3 4	
P125	1012,54	Ba		2 4 4 1	
P126	1098,53	B		2 4 4 2	
P127	1045,59	B		2 4 4 3	
P128	1064,57	A		2 4 4 4	
P129	855,54	Ab		3 1 1 1	
P130	941,53	Ab		3 1 1 2	

Peptoid	Molecular weight	Classification with letter code	Classification with color code	Sidechain with number code	Sidechain with color code
P131	888,59	Cab		3 1 1 3	
P132	907,57	A		3 1 1 4	
P133	941,53	A		3 1 2 1	
P135	974,58	Ab		3 1 2 3	
P136	993,56	Cab		3 1 2 4	
P137	888,59	B		3 1 3 1	
P139	921,64	B		3 1 3 3	
P140	940,62	C		3 1 3 4	
P141	907,57	Bc		3 1 4 1	
P142	993,56	Bc		3 1 4 2	
P144	959,6	B		3 1 4 4	
P145	941,53	B		3 2 1 1	
P146	1027,52	B		3 2 1 2	
P147	974,58	Ca		3 2 1 3	
P148	993,56	Cb		3 2 1 4	
P149	1027,52	Bc		3 2 2 1	
P150	1113,51	B		3 2 2 2	
P151	1060,57	Ca		3 2 2 3	
P152	1079,55	B		3 2 2 4	
P153	974,58	Bc		3 2 3 1	
P154	1060,57	Cb		3 2 3 2	
P155	1007,63	Ca		3 2 3 3	
P156	1026,61	B		3 2 3 4	
P157	993,56	B		3 2 4 1	
P158	1079,55	Cb		3 2 4 2	
P159	1026,61	Cb		3 2 4 3	
P160	1045,59	Bc		3 2 4 4	
P161	888,59	B		3 3 1 1	
P162	9974,58	B		3 3 1 2	
P163	921,64	B		3 3 1 3	
P165	974,58	C		3 3 2 1	
P166	1060,57	B		3 3 2 2	
P167	1007,63	B		3 3 2 3	
P168	1026,61	Bac		3 3 2 4	
P169	921,64	Cb		3 3 3 1	
P170	1007,63	Cb		3 3 3 2	
P171	954,69	Bac		3 3 3 3	

Peptoid	Molecular weight	Classification with letter code	Classification with color code	Sidechain with number code	Sidechain with color code
P172	973,667	Ab		3 3 3 4	
P174	1026,61	Ca		3 3 4 2	
P175	973,67	B		3 3 4 3	
P176	992,65	Ab		3 3 4 4	
P177	907,57	Ab		3 4 1 1	
P178	993,56	Ab		3 4 1 2	
P179	940,62	B		3 4 1 3	
P180	959,6	Ab		3 4 1 4	
P182	7079,55	Ba		3 4 2 2	
P183	1026,61	Ba		3 4 2 3	
P184	1045,59	Ab		3 4 2 4	
P185	940,62	Ab		3 4 3 1	
P186	1026,61	Ba		3 4 3 2	
P187	973,67	Ba		3 4 3 3	
P188	992,65	Ba		3 4 3 4	
P189	959,6	Ab		3 4 4 1	
P190	1045,59	Ab		3 4 4 2	
P191	992,65	Ba		3 4 4 3	
P192	1011,63	Ab		3 4 4 4	
P193	874,52	Ba		4 1 1 1	
P194	960,51	Ba		4 1 1 2	
P195	907,57	Ab		4 1 1 3	
P196	926,55	Ba		4 1 1 4	
P197	960,51	A		4 1 2 1	
P198	1046,5	Ab		4 1 2 2	
P199	993,56	B		4 1 2 3	
P200	1012,54	A		4 1 2 4	
P201	907,57	Ba		4 1 3 1	
P202	993,56	Ba		4 1 3 2	
P203	940,62	Ab		4 1 3 3	
P204	959,6	B		4 1 3 4	
P205	926,55	B		4 1 4 1	
P206	1012,54	Ab		4 1 4 2	
P207	959,6	A		4 1 4 3	
P208	978,58	A		4 1 4 4	
P209	960,51	Ab		4 2 1 1	
P210	1046,5	Bc		4 2 1 2	

Peptoid	Molecular weight	Classification with letter code	Classification with color code	Sidechain with number code	Sidechain with color code
P211	993,56	Ca		4 2 1 3	
P212	1012,54	Ba		4 2 1 4	
P213	1046,5	Ca		4 2 2 1	
P214	1132,49	Cab		4 2 2 2	
P215	1079,55	Ca		4 2 2 3	
P216	1098,53	Ba		4 2 2 4	
P217	993,56	Cab		4 2 3 1	
P218	1079,55	Cab		4 2 3 2	
P219	1026,61	Ab		4 2 3 3	
P220	1045,59	Ac		4 2 3 4	
P221	1012,54	Cb		4 2 4 1	
P222	1098,53	B		4 2 4 2	
P223	1045,59	Ab		4 2 4 3	
P225	907,57	A		4 3 1 1	
P226	993,56	A		4 3 1 2	
P227	940,62	A		4 3 1 3	
P228	959,6	B		4 3 1 4	
P229	993,56	A		4 3 2 1	
P230	1079,55	C		4 3 2 2	
P232	1045,59	A		4 3 2 4	
P233	940,62	A		4 3 3 1	
P234	1026,61	C		4 3 3 2	
P236	992,65	A		4 3 3 4	
P237	959360	B		4 3 4 1	
P238	1045,59	A		4 3 4 2	
P239	992,65	A		4 3 4 3	
P240	1011,63	A		4 3 4 4	
P241	926,55	Ab		4 4 1 1	
P242	1012,54	A		4 4 1 2	
P243	959,6	B		4 4 1 3	
P244	978,58	B		4 4 1 4	
P245	1012,54	A		4 4 2 1	
P246	1098,53	B		4 4 2 2	
P247	1045,59	A		4 4 2 3	
P248	1064,57	Ac		4 4 2 4	
P249	959,6	Ba		4 4 3 1	
P250	1045,59	B		4 4 3 2	

Peptoid	Molecular weight	Classification with letter code	Classification with color code	Sidechain with number code	Sidechain with color code
P251	992,65	Ab		4 4 3 3	
P252	1011,63	B		4 4 3 4	
P253	978,58	Ba		4 4 4 1	
P255	1011,63	B		4 4 4 3	
P256	1030,61	Ca		4 4 4 4	

Table 3.2 The classification of CPPo based on its distribution in BY-2 cells

The first column stands for each individual CPPo in the library based on its serial number. The second column stands for each molecular weight. The third column stands for its classification base on distribution pattern, A, a and colour black stand for vacuole pattern, B, b and colour blue stand for dot pattern, C, c and colour orange stand for membrane pattern. The fourth and fifth column stand for two methods of coding CPPo shown in figure 3.4

Three patterns discussed above are the major distribution pattern of CPPo in BY-2 cells. However, no distinguish line were among these three patterns. In most of the situation, a CPPo had two even all three traits in its distribution pattern. Thus, every CPPo was classified with one major distribution pattern and two minor ones. In column classification with letter code in table 3.2, capital letters represent the major trait of this CPPo while the lower case letters represent the minor traits. In the column classification with color code, color of the first sub-column indicates the major trait and the latter two the minor ones. In columns the side chain with number code and side chain with color code, the structure of CPPo are shown. In next part of this thesis, the relationship between the CPPo distribution patterns and chemical structures are examined.

3.1.4 The linkage between CPPo distribution pattern in cell and the chemical structure

As described in 3.1.3, different CPPos in the library could be classified into three categories due to their different distribution patterns: vacuole pattern, dot pattern and membrane pattern. But the reasons why these chemically similar peptoids distributed so differently inside BY-2 cells are unclear. Since the major difference between individual in the library is the different combination of side chains and the possible different spacial structures, the linkage between the distribution pattern in cell and the chemical structures of CPPo have been explored.

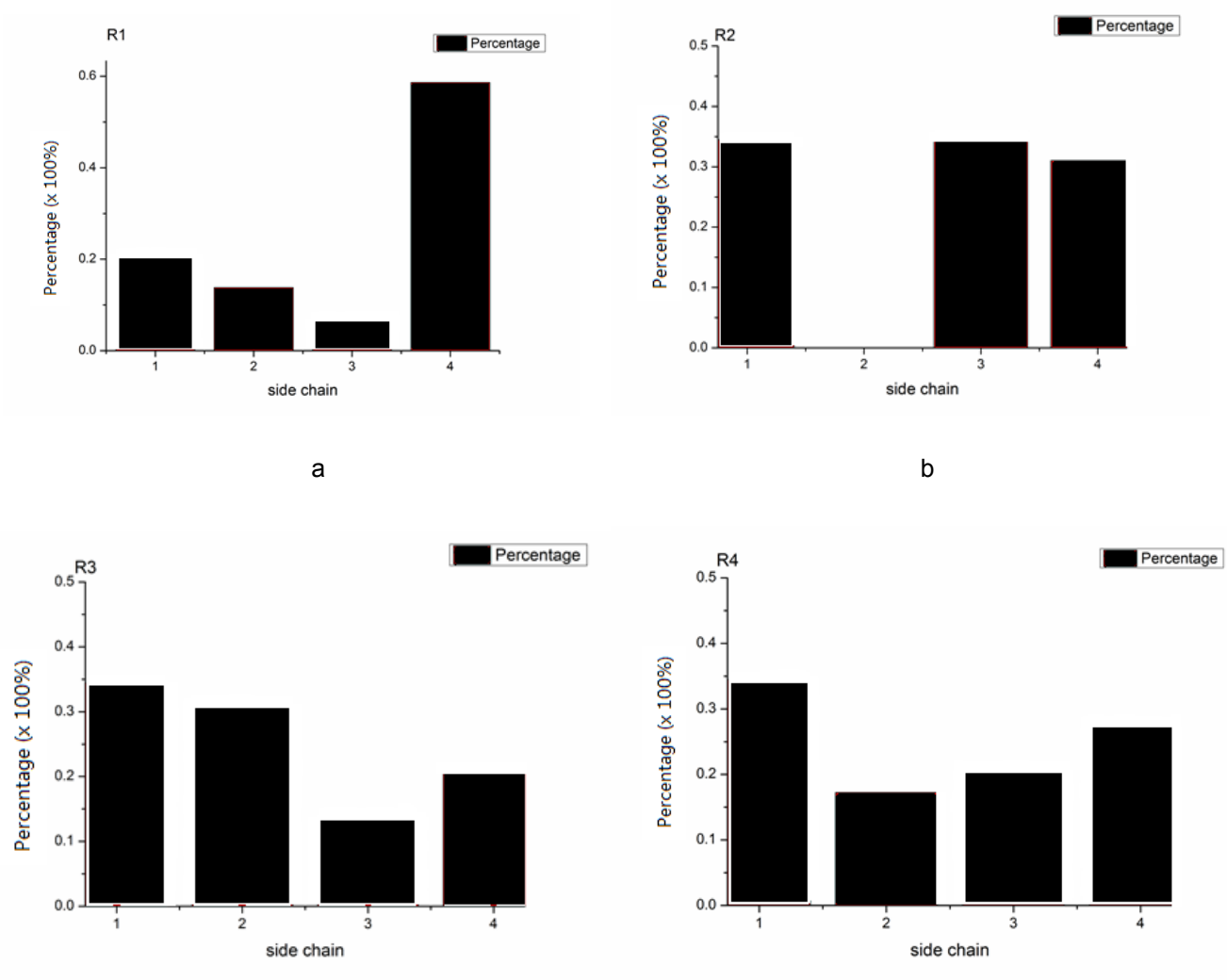


Figure 3.5 The R residue preference of vacuole pattern CPPo

The Rx in left top corner means the R residue position, the number on X axis means the number of chemical structure of side chain displayed in figure 3.4

The R residue chemical structures of all vacuole pattern CPPo have been inspected and counted. Based on the chemical preference of each R residue position, figure 3.5 were charted. From figure 3.5 it could tell: in R1 position chemical structure 4 is the most favourite side chain choice, in R2 position chemical structure 3 is slightly more favourite than structure 1, in R3 position chemical structure 1 is most favourite, and in R4 position chemical structure 1 is also the most favourite one. R1 and R4 positions show lipophilic affinity preference. Thus, theoretically the optimise side chain chemical structure of a vacuole pattern distributed CPPo is 4311. Cross checked with table 3.2, P225 with the chemical structure 4311 distributed in vacuole pattern.

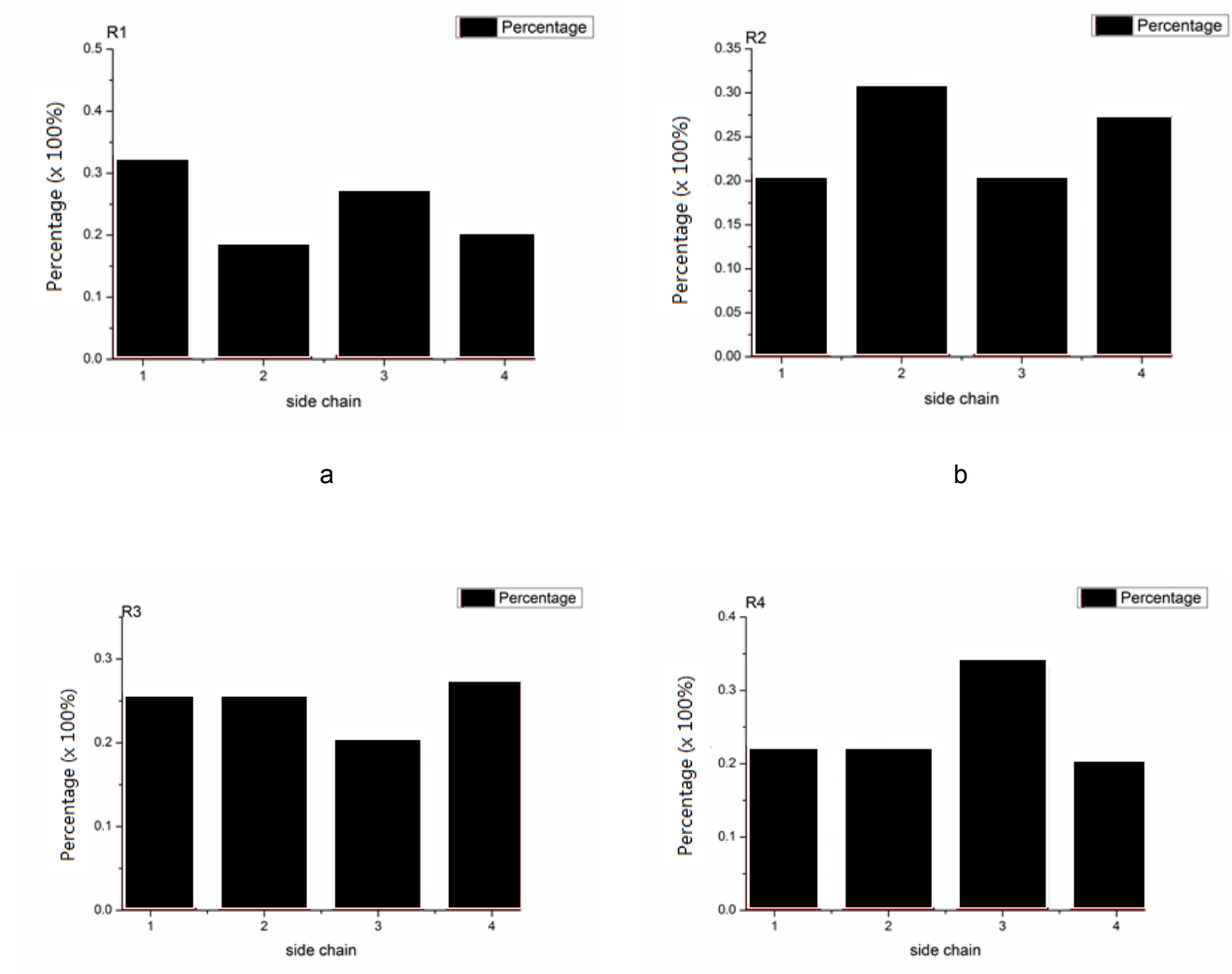


Figure 3.6 The R residue preference of dot pattern CPPo

The Rx in left top corner means the R residue position, the number on X axis means the number of chemical structure of side chain displayed in figure 3.4

Same method described above have been applied to dot pattern distributed CPPo. Based on the chemical preference of each R residue position, figure 3.6 were charted. From

figure 3.6 it could tell: in R1 position chemical structure 1 is the most favourite side chain choice, in R2 position chemical structure 2 is the most favourite one, in R3 position chemical structure 4 shows a little bit better affinity to structure 1 and 2, in R4 position chemical structure 3 is the most favourite one. R2 shows strong lipophilic affinity preference and R4 shows hydrophilic affinity preference. Thus, theoretically the optimise chemical structure of a dot pattern distributed CPPo is 1243. Cross checked with table 3.2, P31 with the chemical structure 1243 distributed in membrane pattern.

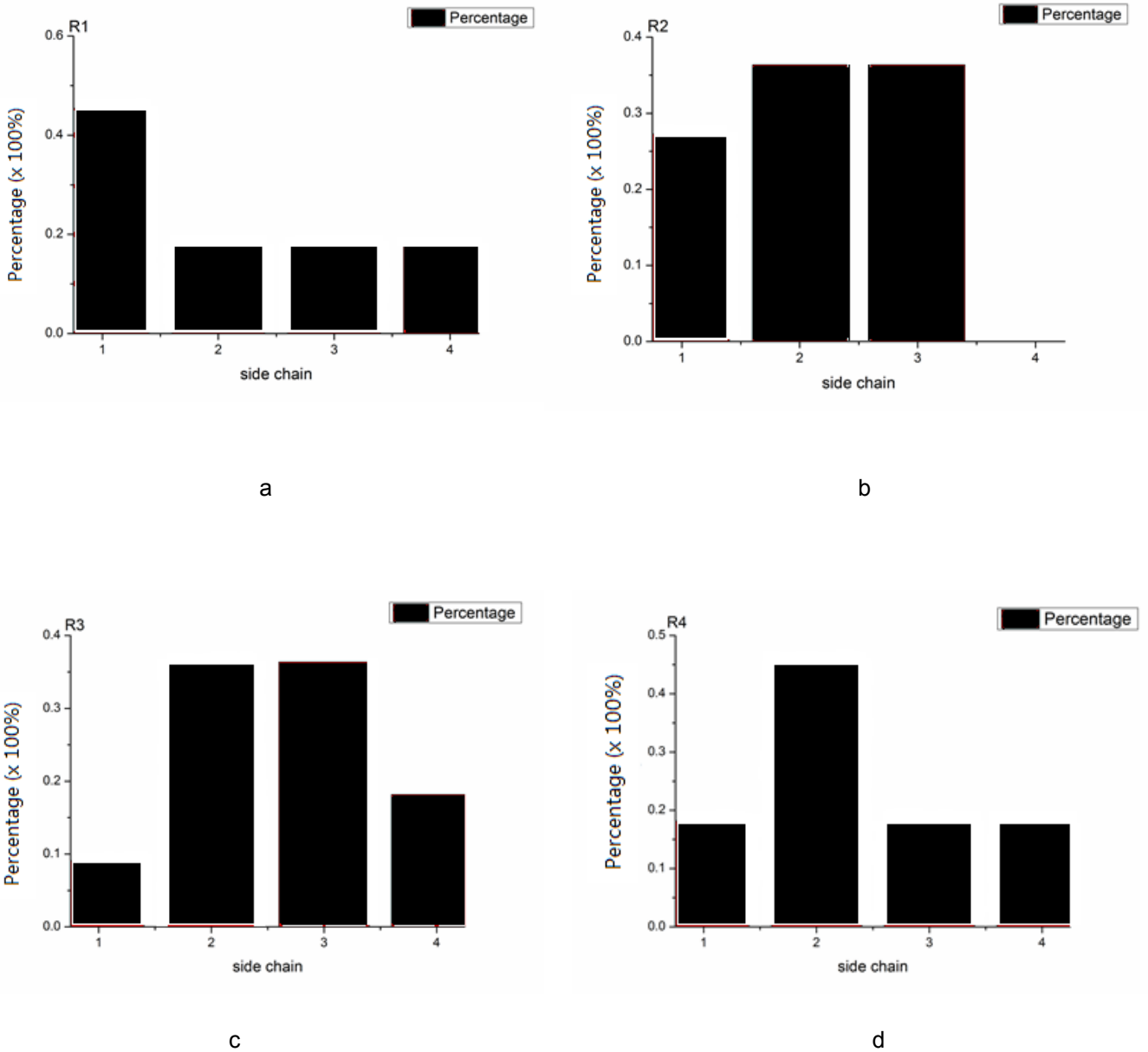


Figure 3.7 The R residue preference of membrane pattern CPPo

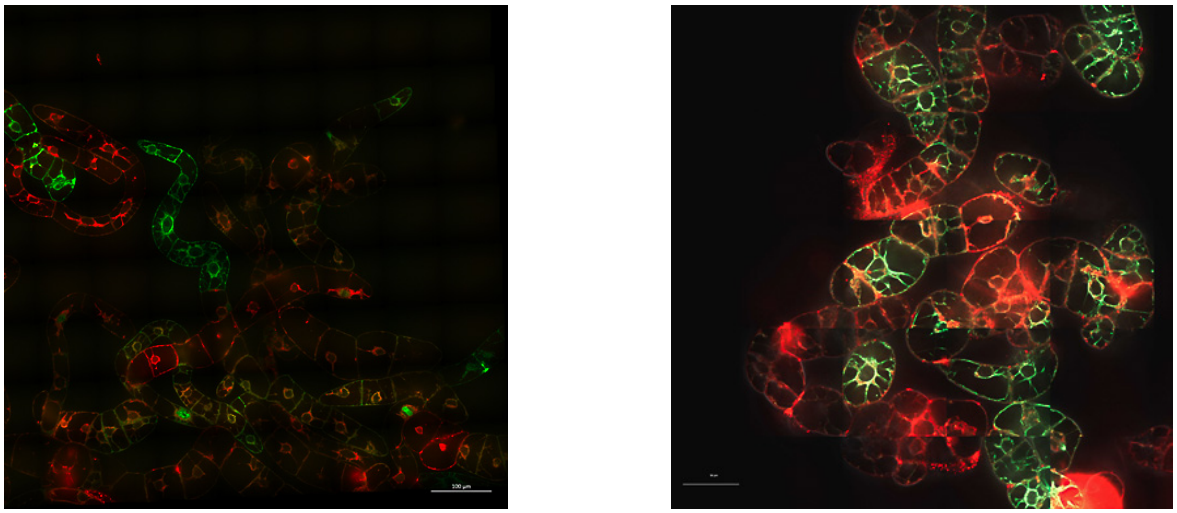
The Rx in left top corner means the R residue position, the number on X axis means the number of chemical structure of side chain displayed in figure 3.4

The R residue chemical structures of all membrane pattern CPPo have been inspected and counted. Based on the chemical preference of each R residue position, figure 3.7 were charted. From figure 3.7 it could tell: in R1 position chemical structure 1 is the most favourite side chain choice, in R2 position chemical structure 2 and 3 are equally favourite, 4 is the least favourite one, in R3 position chemical structure 2 and 3 are also equally favourite, in R4 position chemical structure 2 is the most favourite one. R1 shows weak lipophilic affinity preference and R 4 shows strong lipophilic affinity preference. Thus, theoretically the optimise chemical structure of a membrane pattern distributed CPPo is 1232,1332,1222 and 1322. Cross checked with table 3.2, only P26 with the chemical structure 1232 distributed in membrane pattern.

3.2 CPPo cytology studies

3.2.1 Cytoskeleton colocalization

There are two kinds of cytoskeletons inside plant cells, microtubule and actin. Cytoskeletons have multiple biological functions such as providing mechanism force and holding physical shape of cells, signal transduction, intracellular transport, etc (Alberts et al., 2008). Therefore if any of CPPo in this library could colocalization with cytoskeleton, this CPPo would be a perfect candidate for future industry modifications.



a b

Figure 3.8. The cytoskeleton and CPPo colocalization

a is Tuβ6 cell treated with CPPo P52, showing the microtubule and CPPo P52 colocalization. b is GF11 cell treated with CPPo P128, showing the actin and CPPo P128 colocalization.

Figure 3.8 shows the results of cytoskeleton and CPPo colocalization experiments. a is the result of Tuβ6 cell treated with CPPo P52, showing that the P52 and microtubule's location inside cell. b is the result of GF11 cell treated with CPPo P128, showing that the P128 and actin filament's location inside cell. Looking at the detail of these two pictures, the red signal is CPPo, the green signal is cytoskeleton. However, there are nearly no yellow signal indicate that CPPo and cytoskeleton colocalized together. There are a few yellow signals in both pictures, but the reason for this is because CPPo and cytoskeleton were accidentally in one place, not because they were colocalized.

3.2.2 Actin inhibitor latrunculin B treatment

Latrunculin B is an actin inhibitor, so after the disruption of actin filament inside tobacco WT cells, CPPo distribution patterns change were expected.

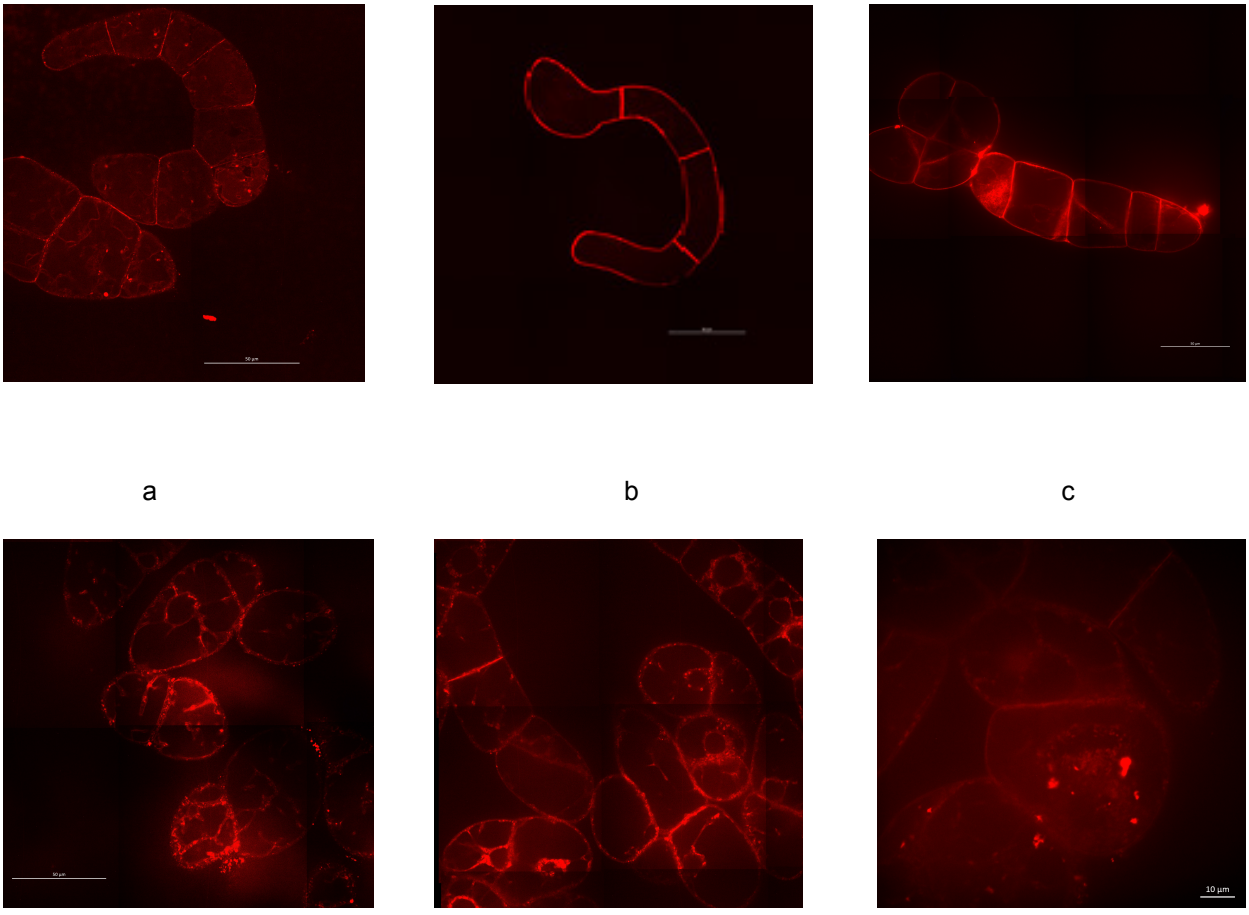


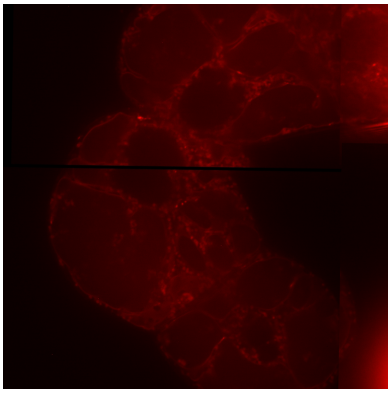
Figure 3.9 Latrunculin B and CPPo treated cells

a and e are P37 treated cells, b and f are P165 treated cells, c and g are P180 treated cells. a, b, c are Latruncunlin B and treated cells. e, f, and g are only CPPo treated control group.

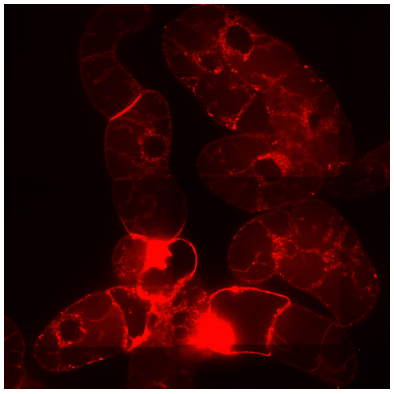
In this assay, P180 represent vacuole pattern, P165 represent membrane pattern, P37 represent dot pattern. In pictures a and e, P37 still holds dot pattern. In pictures b and f, P165 still holds membrane pattern. In picture c and g, P180 still spreads like a cloud and holds the vacuole pattern. This result suggests that the distribution of CPPo in this library has nothing to do with actin filament.

3.2.3 Microtubule inhibitor oryzalin

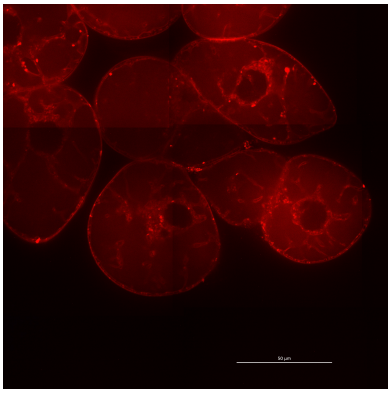
Like colchicine, oryzalin could interrupt tubulin assemble and make microtubule disassembled. So when BY-2 cells applied with oryzalin and CPPo, CPPo distribution pattern may change.



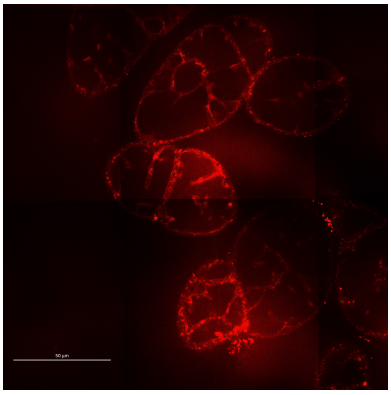
a



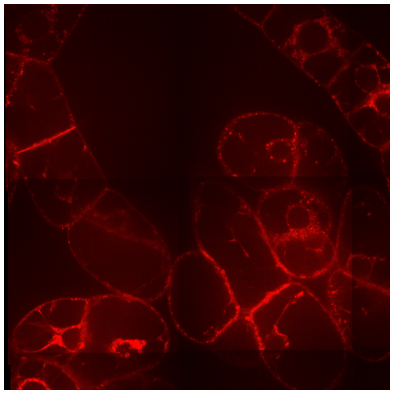
b



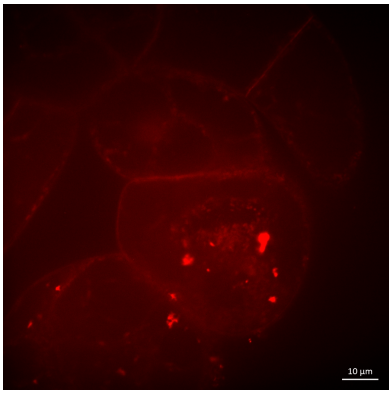
c



e



f



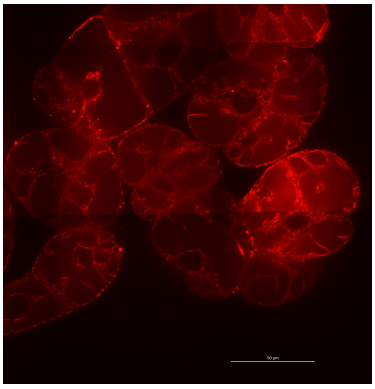
g

Figure 3.10 Oryzalin and CPPo treated cells
a and e are P37 treated cells, b and f are P165 treated cells, c and g are P180 treated cells. a, b, c are Oryzalin and treated cells. e, f, and g are only CPPo treated control group.

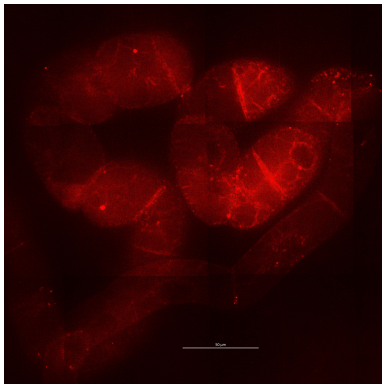
Like Latrucunlin B assay, in this assay P180 also represent vacuole pattern, P165 membrane pattern and P37 dot pattern. In pictures a and e, P37 still holds dot pattern. In pictures b and f, P165 still holds membrane pattern. In picture c and g, P180 still spreads like a cloud and holds the vacuole pattern. This result suggests that the distribution of CPPo in this library has nothing to do with microtubule.

3.2.4 Myosin inhibitor BDM

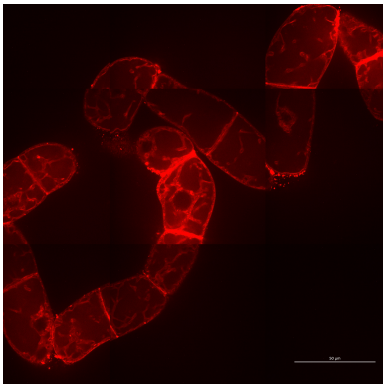
2,3-butanedione monoxime, BDM for short, is a myosin inhibitor. In this assay, the relationship between CPPo distribution and myosin has been investigatigated.



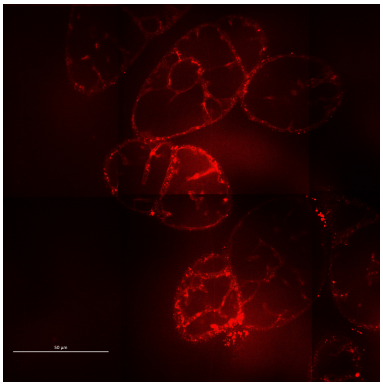
a



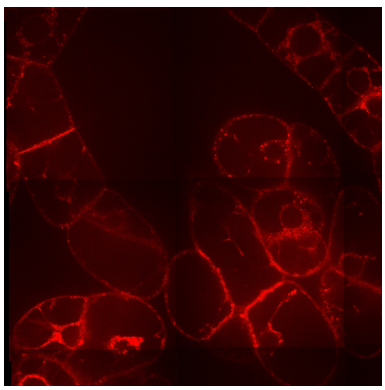
b



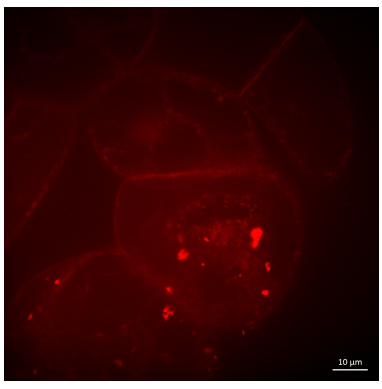
c



e



f



g

Figure 3.11 BDM and CPPo treated cells
a and e are P37 treated cells, b and f are P165 treated cells, c and g are P180 treated cells. a, b, c are BDM and treated cells. e, f, and g are only CPPo treated control group.

Like two experiments above, in this assay P180 also represent vacuole pattern, P165 membrane pattern and P37 dot pattern. In pictures a and e, P37 still holds dot pattern. In pictures b and f, P165 still holds membrane pattern, but some dots appears after treatment with BDM. In picture c and g, P180 still spreads like a cloud and holds the vacuole pattern, but some CPPos begin to accumulate on cell membrane. This result suggests that the distribution of CPPo in this library may have something to do with physiology functions of myosin.

3.2.5 CPPo and mitochondria colocolization

Mito Tracker Green can attach to mitochondria and label it with green fluorescent signal under the microscope. With the help of it, a dot pattern distributing CPPo P127's location inside of BY-2 cells has been investigated.

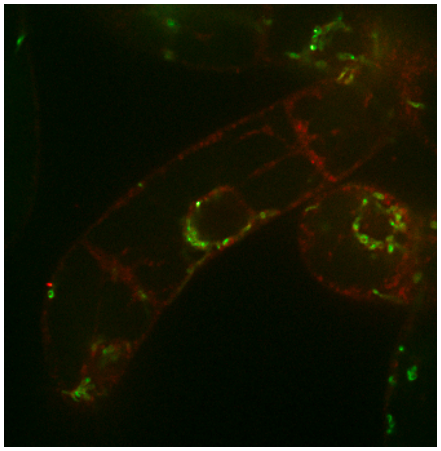


Figure 3.12 P127 and mitochondria colocolization assay

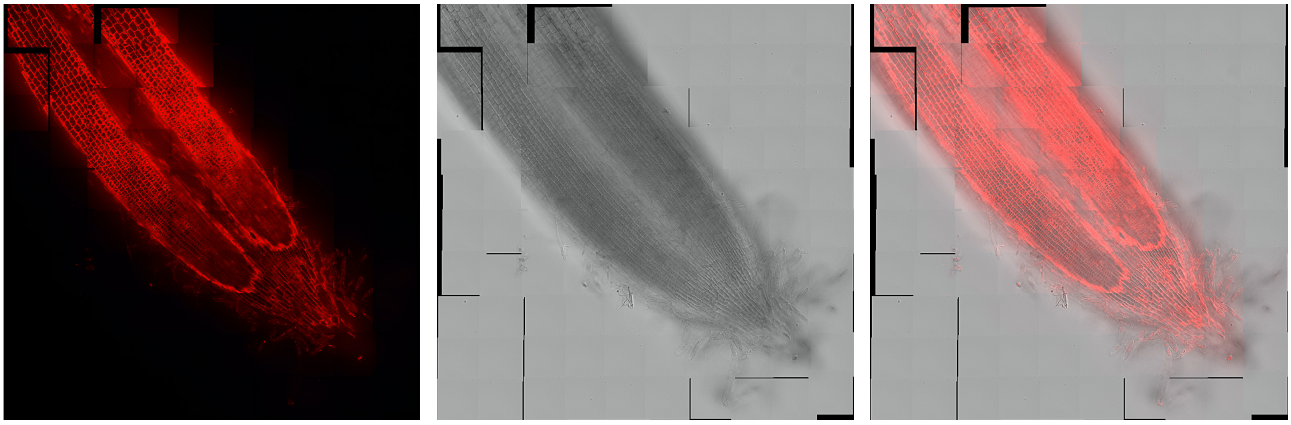
As showing in figure 3.12, the green dots are mitochondria marked with Mito Tracker Green, and the red dots indicate the location of CPPo P127. No yellow dot in this picture suggests that CPPo P127 could not colocolized with mitochondria.

3.3 The CPPo library's behaviour in rice root tip

As an engineering oriented research, CPPo library are hoped to be put into application in future. No matter in scientific field or industry field, individual hierarchy is a basic level that life activities perform as one single organism. Thus the behaviour of CPPos on individual hierarchy may influence the metabolism of the whole plant. Therefore, two aspects of CPPo should be learned. First, whether these CPPo have particular distribution pattern in tissue or organ level. Second, if they do have patterns, whether these pattern have biology meaning, or at least whether these CPPo are safe.

3.3.1 The CPPo's penetration ability in rice root tip

Although the penetration ability of CPPo in suspension BY-2 cells has been tested, but whether they could penetrate into real plant cells is still a question. So before the utilisation of CPPo in rice could be investigated, their penetration ability has been tested.



a b c

Figure 3.13 CPPo P114 inside of rice seedling root tip
a is a RFP picture, b is a DIC picture, c is a merge picture of two channels

After 2 hours incubation in 2 μM CPPo, rice root tip has been observed under spinning disc microscope. Picture of P114 are shown in figure 3.13. The red signals in figure 3.13 a indicate that CPPo in the library could penetrate in rice tip cells in vivo.

3.3.2 Distribution of CPPo inside rice root tip

Like their behaviour inside of suspension BY-2 cells, during the process of screening, by the help of Zen, two major distribution pattern have been found. Pattern A: CPPo could

penetrate in all cells in the root tip. Pattern B: CPPo only located on the wall of vascular bundles.

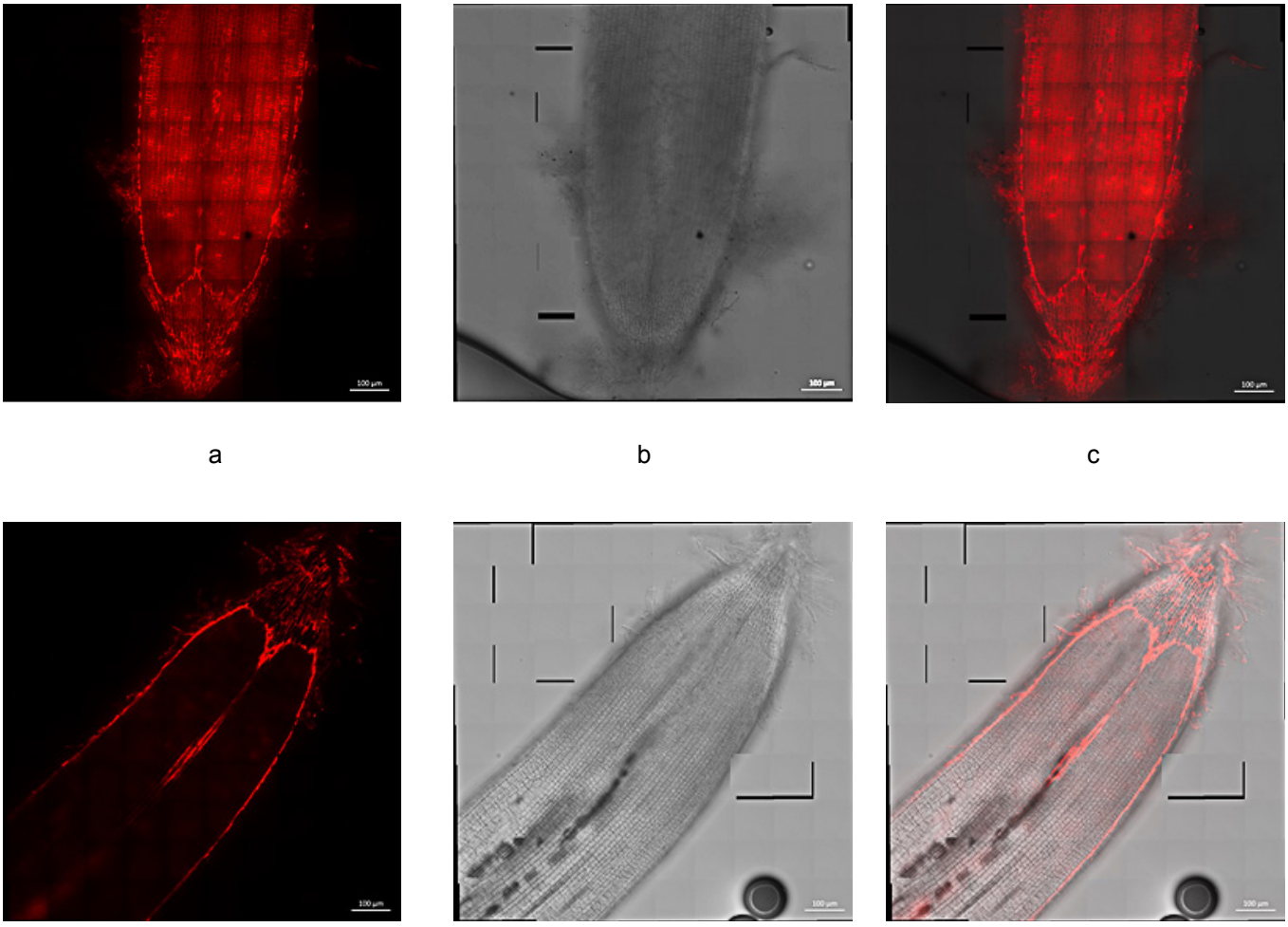
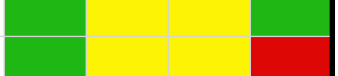
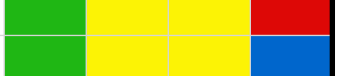
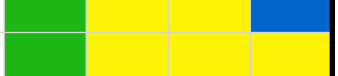
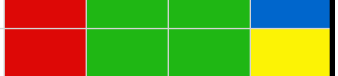


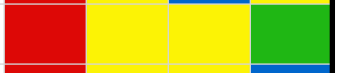
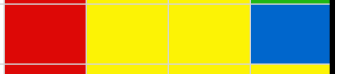
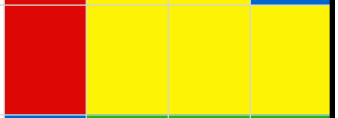
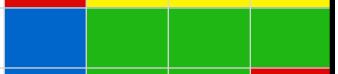
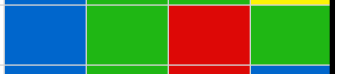
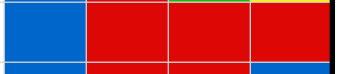
Figure 3.14 The distribution pattern of CPPo in rice root tip
a, b and c are pictures of P5 which is a A pattern CPPo , e, f and g are pictures of P96 which is a B pattern CPPo. a and e are RFP pictures, b and f are DIC pictures, c and g are merge pictures. The explore time of b has been increased by Lightroom in order to get a clear profile of root tip.

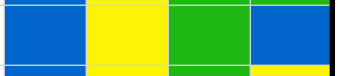
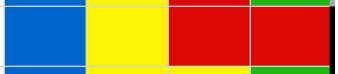
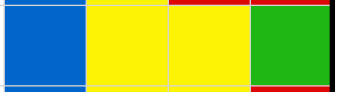
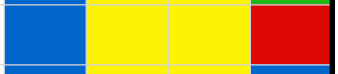
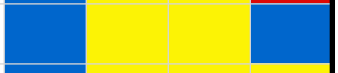
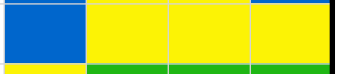
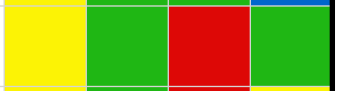
The results of CPPo classification based on distribution patterns are show in table 3.3. Most of CPPo belong to Pattern A, means that they could penetrate in nearly all cells in the rice root tip. A small amount of CPPo in this library belong to Pattern B. However there are a few of CPPo have multiple distribution patterns, and these traits are marked in letter code column in table 3.3.

Peptoid	Molecular weight	letter code	color code	Sidechain with number code	Sidechain with color code
P1	822,49	A		1 1 1 1	
P3	855,55	A		1 1 1 3	
P4	874,52	B		1 1 1 4	
P5	908,48	A		1 1 2 1	
P6	994347	A		1 1 2 2	
P8	960,51	A		1 1 2 4	
P9	855,54	B		1 1 3 1	
P10	941,53	A		1 1 3 2	
P11	888,59	A		1 1 3 3	
P13	874,52	B		1 1 4 1	
P14	960,51	B		1 1 4 2	
P15	907,57	A		1 1 4 3	
P17	908,48	B		1 2 1 1	
P18	994,47	A		1 2 1 2	
P19	941,53	A		1 2 1 3	
P20	960,51	B		1 2 1 4	
P21	994,47	A		1 2 2 1	
P22	1080,46	B		1 2 2 2	
P23	1029,52	B		1 2 2 3	
P24	1046,5	A		1 2 2 4	
P25	941,53	B		1 2 3 1	
P26	1027,52	B		1 2 3 2	
P27	974,58	B		1 2 3 3	
P28	993,56	A		1 2 3 4	
P29	960,51	A		1 2 4 1	
P30	1046,5	A		1 2 4 2	
P33	855,54	A		1 3 1 1	
P35	888,59	A		1 3 1 3	
P36	907,57	B		1 3 1 4	
P37	941,53	A		1 3 2 1	

P38	1027,52	A		1 3 2 2	
P40	993,56	A		1 3 2 4	
P41	888,59	A		1 3 3 1	
P42	974,58	A		1 3 3 2	
P44	940,62	A		1 3 3 4	
P45	907,57	A		1 3 4 1	
P46	993,56	A		1 3 4 2	
P47	940,62	A		1 3 4 3	
P48	959.6	B		1 3 4 4	
P49	874,52	A		1 4 1 1	
P50	960,51	A		1 4 1 2	
P51	907,57	A		1 4 1 3	
P52	926,55	A		1 4 1 4	
P54	1046,5	A		1 4 2 2	
P55	993,56	A		1 4 2 3	
P56	1012,54	A		1 4 2 4	
P58	993,56	A		1 4 3 2	
P59	940,62	A		1 4 3 3	
P60	959,6	A		1 4 3 4	
P61	926,55	A		1 4 4 1	
P62	1012,54	A		1 4 4 2	
P63	959,6	A		1 4 4 3	
P64	978,58	A		1 4 4 4	
P66	994,47	A		2 1 1 2	
P67	941,53	A		2 1 1 3	
P68	960,51	A		2 1 1 4	
P70	1080,46	B		2 1 2 2	
P71	1027,52	A		2 1 2 3	
P72	1046,5	A		2 1 2 4	
P73	941,53	A		2 1 3 1	
P75	974,58	A		2 1 3 3	
P76	993,56	A		2 1 3 4	

P79	993,56	A		2 1 4 3				
P80	1012,54	A		2 1 4 4				
P81	994,47	A		2 2 1 1				
P82	1080,46	A		2 2 1 2				
P83	1027,52	B		2 2 1 3				
P84	1046,5	B		2 2 1 4				
P85	1080,46	B		2 2 2 1				
P86	1166,45	B		2 2 2 2				
P87	1113,51	A		2 2 2 3				
P89	1027,52	A		2 2 3 1				
P90	1113,51	A		2 2 3 2				
P91	1060,57	A		2 2 3 3				
P92	1079,55	B		2 2 3 4				
P93	1046,5	A		2 2 4 1				
P94	1132,49	A		2 2 4 2				
P95	1079,55	A		2 2 4 3				
P96	1098,53	B		2 2 4 4				
P98	1027,52	A		2 3 1 2				
P99	974,58	A		2 3 1 3				
P100	993,56	A		2 3 1 4				
P101	1027,52	A		2 3 2 1				
P102	1113,51	A		2 3 2 2				
P104	1079,55	A		2 3 2 4				
P105	974,58	A		2 3 3 1				
P106	1060,57	A		2 3 3 2				
P107	1007,63	A		2 3 3 3				
P109	993,56	A		2 3 4 1				
P110	1079,55	A		2 3 4 2				
P111	1026,61	A		2 3 4 3				
P113	960,51	A		2 4 1 1				
P114	1046,5	A		2 4 1 2				
P115	993,56	A		2 4 1 3				

P116	1012,54	A		2 4 1 4	
P117	1046,5	B		2 4 2 1	
P118	1132,49	B		2 4 2 2	
P119	1079,55	A		2 4 2 3	
P123	1026,61	A		2 4 3 3	
P124	1045,59	A		2 4 3 4	
P125	1012,54	A		2 4 4 1	
P127	1045,59	A		2 4 4 3	
P128	1064,57	A(not on the membrane)		2 4 4 4	
P129	855,54	A		3 1 1 1	
P130	941,53	A		3 1 1 2	
P131	888,59	B		3 1 1 3	
P132	907,57	A		3 1 1 4	
P133	941,53	A		3 1 2 1	
P135	974,58	A		3 1 2 3	
P136	993,56	A		3 1 2 4	
P138	974,58	A		3 1 3 2	
P139	921,64	A		3 1 3 3	
P140	940,62	A		3 1 3 4	
P141	907,57	A(not on the membrane)		3 1 4 1	
P142	993,56	A		3 1 4 2	
P144	959,6	A		3 1 4 4	
P145	941,53	B		3 2 1 1	
P146	1027,52	A		3 2 1 2	
P147	974,58	A		3 2 1 3	
P148	993,56	B		3 2 1 4	
P150	1113,51	A		3 2 2 2	
P151	1060,57	A		3 2 2 3	
P152	1079,55	A		3 2 2 4	
P154	1060,57	B		3 2 3 2	

P155	1007,63	A		3 2 3 3	
P156	1026,61	B		3 2 3 4	
P157	993,56	A		3 2 4 1	
P159	1026,61	A		3 2 4 3	
P160	1045,59	A(but like pattern B)		3 2 4 4	
P161	888,59	A		3 3 1 1	
P163	921,64	A		3 3 1 3	
P166	1060,57	A		3 3 2 2	
P167	1007,63	A		3 3 2 3	
P168	1026,61	A(but like pattern B)		3 3 2 4	
P169	921,64	B		3 3 3 1	
P170	1007,63	A		3 3 3 2	
P172	973,667	B		3 3 3 4	
P174	1026,61	A		3 3 4 2	
P176	992,65	A		3 3 4 4	
P177	907,57	B		3 4 1 1	
P179	940,62	B		3 4 1 3	
P180	959,6	A		3 4 1 4	
P181	993,56	A		3 4 2 1	
P182	7079,55	A		3 4 2 2	
P189	959,6	A(but like pattern B)		3 4 4 1	
P190	1045,59	A		3 4 4 2	
P191	992,65	A		3 4 4 3	
P192	1011,63	B		3 4 4 4	
P193	874,52	A		4 1 1 1	
P195	907,57	A		4 1 1 3	
P197	960,51	A (but like B pattern)		4 1 2 1	
P200	1012,54	A		4 1 2 4	
P201	907,57	B		4 1 3 1	
P203	940,62	A		4 1 3 3	
P205	926,55	A		4 1 4 1	

P207	959,6	B		4 1 4 3				
P211	993,56	A		4 2 1 3				
P214	1132,49	A		4 2 2 2				
P241	926,55	A		4 4 1 1				

Table 3.3 The classification of CPPo based on its distribution in rice seedling root tip
The pattern A is marked orange and pattern B blue. There are some notes if the CPPo distribution is not typical A or B

3.3.3 The CPPo library will not harm the rice seedling

Although CPPo in this library can penetrate into rice root tip very well, and show certain distribution pattern, but if they do harm to the plant, their future usage in agriculture are limited. Therefore, a short-term growth assay has been performed as described in 2.5.4.

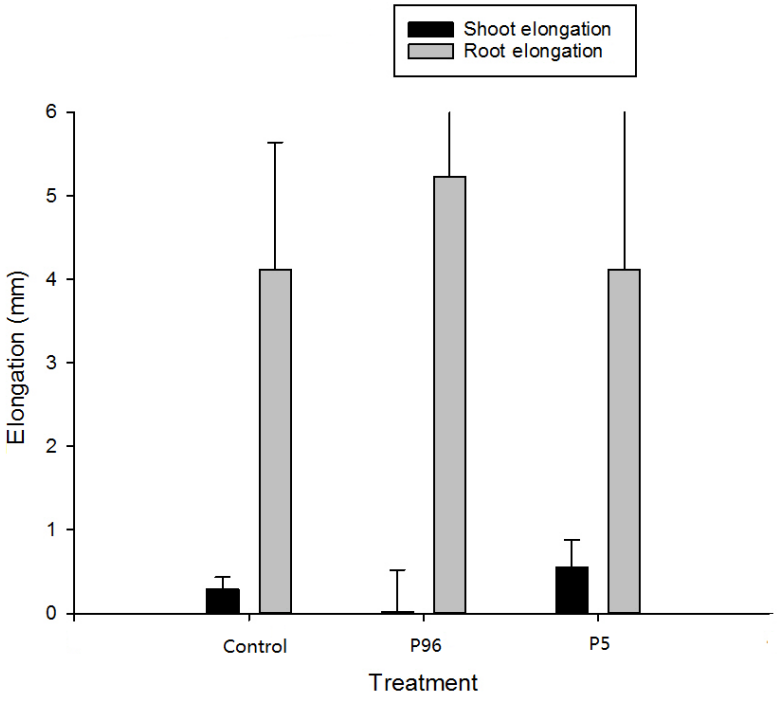


Figure 3.14. The elongation length of rice seedling root and shoot
The black bar is shoot elongation length and the grey bar is root elongation length

Two CPPo were selected in this assay, P96 represent Pattern B and P5 represent Pattern A. 50 rice seedlings were used in each treatment and Compare to the control group in Figure 3.14, P96 treated rice seedlings grow faster in root and slower in shoot, P5 treated rice seedlings grow nearly the same speed in root but faster in shoot growth. During this incubator incubation period, all control group rice seedlings, 90% P96 treated seedlings

and 67% P5 treated seedlings expend two leaves and the rest expanded one leaf.

4 Discussion

To get insight into the biological traits of cell penetrating peptoids in this library, they had been tested on both Nicotiana tabacum BY-2 suspension cell system, and observed not only the safety of CPPo to plant cells and the different entry efficiency, but also the different distribution patterns. To further study the CPPo penetration mechanisms and CPPo localization inside cells, several inhibitors and trackers have been applied to CPPo treated BY-2 cells. Furthermore, this library have been applied to Oryza sativa Dongjin seedling root tip system in a short-term fashion to prove the principle of CPPo application possibility in real plant.

4.1 The behaviours of CPPo in the library in suspension BY-2 cells

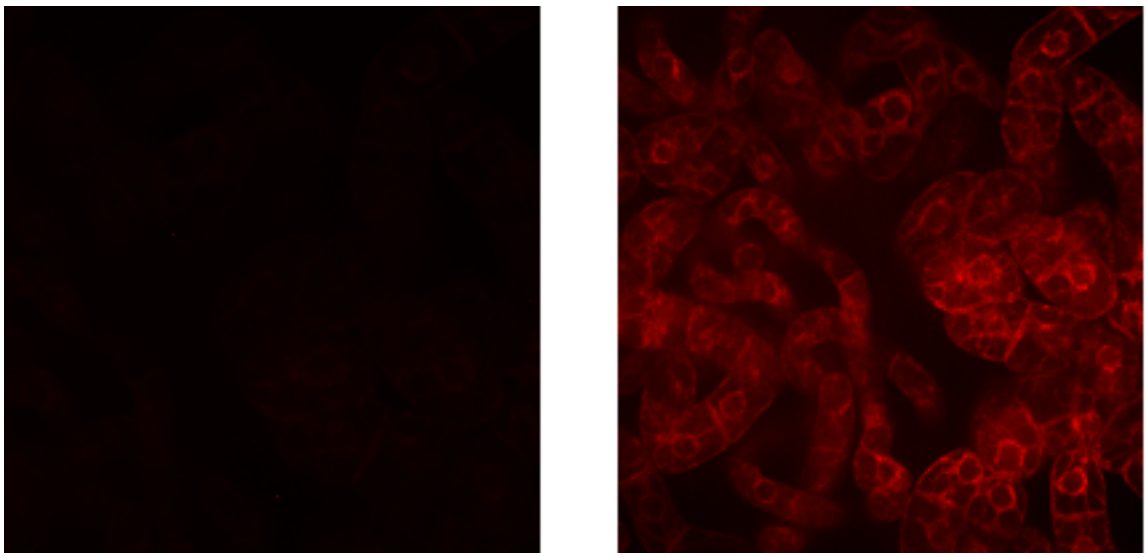
4.4.1 Two foundations of CPPo chemical engineering utilization

There are two major concerns about peptides therapeutical value: 1.the low bio-membrane permeability and 2. the relatively rapid degradation due to the existence of protease (Langel Ü, 2002). However, the first drawbacks have overcome by the finding of CPP, but the second drawback is still a barrier. To overcome it, CPPo were designed and synthesised. With the shift of side chain from α-carbon atom to nitrogen atom, CPP has changed into CPPo (or a new kind of CPPo has been synthesised de novo) and become totally resistant to proteolysis (Miller S M, 1995). But would the newly gained peptoids still penetrate into cells?

Focus on CPPo in the library, they could penetrate into suspension cells but with different efficiency (Figure 3.1). After magnifing the CPPo (labeled with rhodamine) signal in figure 3.1 c, CPPo could be observed inside BY-2 cells (Figure 4.1). This means that regardless of how poor the penetration efficiency is, all CPPo in the library could enter BY-2 cells.

The penetration ability is one pillar to the application of CPPo. But the toxicity is the other pillar. There are many phenotyping indexes, such as mortality, cell file, mitotic index and cell length/width ratio could define the health situation of a cell and colony. In this assay, mortality has been chosen to show the toxicity of CPPo. Besides mortality, other three

indexes are showing the long-term surviving situation of suspension cells, there is no meaning to test them in a short-term assay. As can be seen in figure 3.2, the mortality of selected CPPo (represent different distribution patterns inside cell) treated BY-2 cell is at the same high level (above 95%) of the control group, indicating that CPPo in this library are safe to suspension BY-2 cells, regardless the distribution pattern.



a b

Figure 4.1 Original and enhanced P25 treat BY-2 cell
a is the the original picture of P25 which is also figure 3.2 c, and b is the signal enhanced picture of P25

4.4.2 Three distribution patterns inside cell and their relationship with CPPo chemical structure

As described in 2.3, all CPPos in this library have a universal backbone of four repeat units and the difference among them is the different combination of side chain chemicals at four R-residue sites (Figure 2.1, Figure 2.2). Due to the chemical structural differences, the distribution of CPPo inside of BY-2 cells are different. As shown in figure 3.3, a common feature among these three pattern is that no CPPo signal inside nuclei. That means unlike NLS-based CPP, CPPo in this library could not enter the nuclei due to the lack of nuclear localization sequence. However, the CPPos distributed in vacuole pattern show that they could penetrate in any plasma membrane inside BY-2 cells other than nuclear membrane. Compare to them, the CPPos distributed in dot pattern and membrane pattern could not permeate though vacuole membrane.

Though there are vacuole, dot and membrane three major pattern types, but owning to the minor differences in the chemical structures, there are many CPPo showing a combine

pattern of these major patterns. For example in table 3.2, CPPo P7 showing only vacuole pattern, while CPPo P8 showing both vacuole pattern and dot pattern. The only difference between them is in R4 position, P7 has a side chain 3 and P 8 has a side chain 4 (side chain structure seen in figure 2.2). However sometimes a small change in side chain choices may lead to a huge pattern change such as CPPo P233 and P234. P233 which has a chemical structure 4331 distributes in vacuole pattern and P234 which has a chemical structure 4332 distributes in membrane pattern. The reason for such distribution difference may lays in the 3-D structure difference or hydrophilic/lipophilic changes, but it needs further studies.

However, based on results showing in table 3.2, the relationship between the distribution patterns and the CPPo chemical structures could be roughly discussed. One hypothesis is made: Based on the favourite side chain in each R residue position a optimised CPPo could be found, and it should distribute in the same pattern as the data source. Taking vacuole pattern for example, each R residue position's favourite side chain choices has been displayed in figure 3.5. According to this result, a CPPo P225 containing all four most favourite side chains for four R residue positions is found. Cross check this "optimize" CPPo with table 3.2 it could be found out P225 does distribute in vacuole pattern. However, the result of dot pattern distributing CPPos in figure 3.6 are against the hypothesis. But the result of membrane distributing CPPos in figure 3.7 are partially supporting the hypothesis. This result indicates that the side chain combination is not the only reason that effects CPPo distribution.

4.4.3 Where do CPPos locate inside BY-2 cell?

Knowing the location where CPPo in this library locates could shine lights on the potential target for future chemical or biological engineering. Besides organelles large enough to be observed under microscope like vacuole and nuclei , several possible organelles and proteins has been investigated.

In plant cells, cytoskeleton play important roles in many aspects of biological activities. Thence they become promising targets for engineering. Two cell lines in which cytoskeleton has been fluorescent labeled were treated with CPPo in this library. As shown in figure 3.8, in both mosaic pictures there are red, green and yellow signals. That means that CPPo do not locate on cytoskeleton. The reason for some cells or some parts

of cells showing yellow signal is that because of the vacuole occupied a large amount of space inside cells, as a result all cytoplasm are narrowed into a confined space where many CPPo and cytoskeleton happened to be in the same place.

For dot pattern distributing CPPo, one important question is why do they forming dots or clusters? In other two patterns, CPPo always remain a “dust" shape. So an educated hypothesis is that dot pattern distributing CPPo attached to a kind of dot shape organelle. But according to the result of mitochondria and CPPo colocolization experiment, the CPPo dots do not attach to mitochondria. Another possibility is those dots could be colocalized with peroxisomes, this theory could be tested in future with a peroxisomes marked BY-2 line: OPR7-GFP.

4.4.4 A search for the mechanism of pattern forming

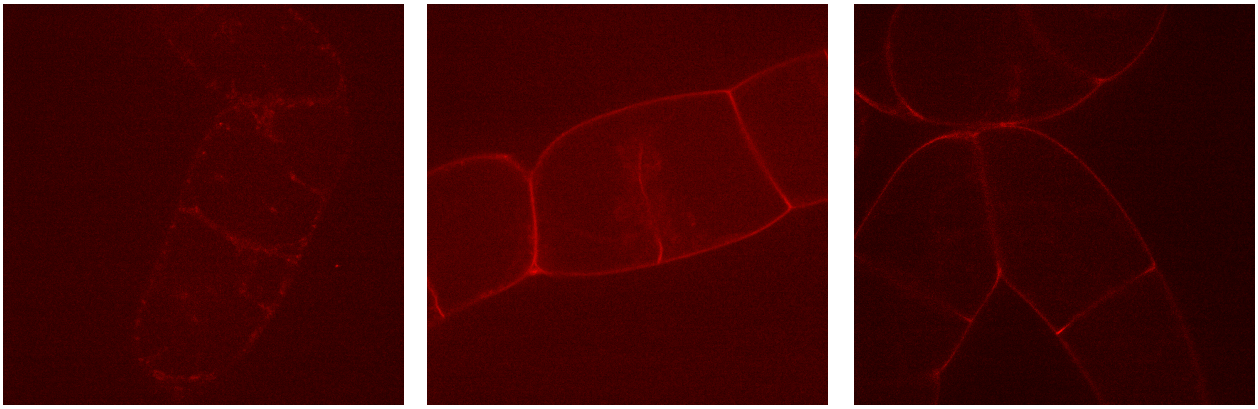
The dot pattern forming CPPo and mitochondria colocalization experiment suggests that mitochondria has nothing to do with the forming of dot pattern. Although the cytoskeleton and CPPo colocalization assay show that no matter microtubule nor actin filaments, neither could colocalized with CPPo, but it does not suggesting that cytoskeleton plays no role in the pattern forming process. Thus, actin inhibitor latrunculin B and microtubule inhibitor oryzalin have been applied to CPPo treated BY-2 cells.

In membrane pattern, most of the CPPo locate on membranes, especially on the membranes linking two neighbouring cells. Though the extra bright signal could be explain by it is actually two signals from neighbouring cells combine together, but it also could be the membrane distributing CPPo in this library could attach to something on the cell membrane separate two cells. One hypothesis about membrane pattern suggests that those CPPo could have certain interaction with PIN protein. PIN protein polarly locates on plasma membrane, and its distribution is in asymmetric way. Therefore, in plant cells it plays a crucial role in the polar auxin transport, forms the auxin directional movement and auxin gradient along the tissue (Ljung et al., 2005; Grieneisen et al., 2007; Robert and Friml, 2009). If this hypothesis is true, after actin be inhibited by LatB, the distribution of PIN protein will be disorder, so does the membrane pattern. However, in figure 3.9 b, compare to figure 3.9 f, P165 still distributed a clear membrane pattern. This result means that the membrane pattern distributing CPPo do not interact with PIN protein.

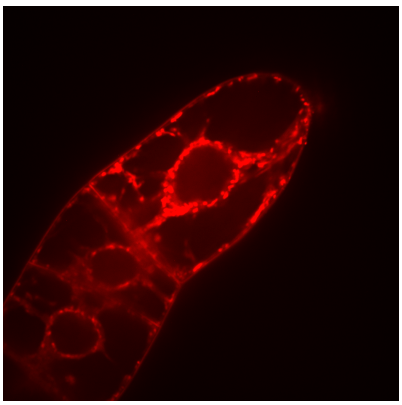
Meanwhile, the other two CPPos distributing two other patterns also remained their original patterns, indicates that actin do not play a role in the distribution of CPPos in this library.

Microtubules play multiple roles inside plant cells: from forming the 3-D shape of cell, signal sensing and inter/intra cellular transportation to forming spindle during mitosis. Yet as the results show in figure 3.10, after BY-2 cells been treated with oryzalin, three CPPos still distributed their patterns faithfully. The reason of a red cluster appearing in the middle bottom of figure 3.10 b is the the cell is dying, and its membrane start to deform, so that CPPo could enter freely with a high speed. This phenomena suggests that the penetrating mechanism of CPPo in this library is not driven by concentration gradient, but a more active (not ATP consuming necessarily) process.

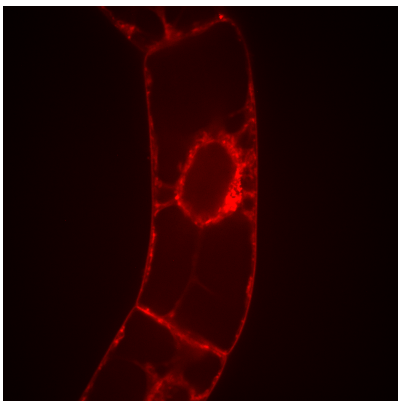
Myosin motors drive a rapid cytoplasmic streaming in plant cells, though the specific mechanism is unknown (Kurth E.G. et al, 2017). Treated with BDM, the ATpase function of myosin has been inhibited. After the treatment, three patterns were all showing some changes. In P37 treated cells, compare to control group, dots are not forming as fast as it should be. In P165 treated cells, clear membrane pattern changed into a mixed pattern of both membrane pattern and vacuole pattern. In P180 treated cells, vacuole pattern seems at the beginning phase (Figure 4.2). Figure 3.11 indicates that the CPPo transportation inside BY-2 cells is a myosin-depend progress.



a b c



d



e

Figure 4.2 Time course of P180 vacuole pattern forming
a is 15 minutes after P180 added, b is 30 minutes, c is 1 hour, d is 1.5 hours and e is 2 hours.

4.2 The behaviours of CPPo in the library in Dongjin seedling root tips

4.2.1 CPPo library could penetrate in rice root tips

As mentioned many time is this thesis, this project is an engineering oriented research. Thus, CPPo must be tested on a real plant for its potential utilisations in industry and agriculture. Rice is a widely grow and important corps that support numerous people in this world, and due to its fast gemination speed, it is chosen as the model system for this short-term assay.

As figure 3.13 indicates, after two hours incubation, CPPo P114 penetrates into cells in the rice seedling root tips. Like in suspension cells, it means one pillar of CPPo application in real plant has been confirmed steady.

The other pillar is the CPPo's toxicity to rice. Data in figure 3.14 show that no matter pattern A distributing CPPo or patter B distributing CPPo, they will not harm the seedling growth, at least no obvious harm have been observed in short-term.

4.2.2 CPPo’s distribution patterns inside rice root tips

Just like in suspension cells, CPPo also show certain patterns inside rice root tips. Figure 3.14 illustrated these two patterns. In pattern A, nearly all cells are filled with CPPo. But in pattern B, only the cap cells, meristem cells and cells on the vascular bundle wall were penetrated. The reason is unknown.

There were also some mixed pattern appear, but much less than in BY-2 cells. Perhaps CPPo tends to show a more clear pattern in tissue level? May the future research answer this question.

5 One Application Example: DOK848, a colchicine transporter could function in BY-2 cell

Although scientific research always explore the boundary of human knowledge, meanwhile correcting the former itself all the time, but the purpose of such exploration is not only limit in knowing the world and fulfil the curiosity of people, but also in using the knowledge gained to created a better world for humanity. Lots of what we learned have been put into application and serve us in both good and bad way, and the art of engineering has been formed. As the development of technology and the invention of new tools, lots of gained knowledge have been abandoned or lost in the tide of history. However, scientist and engineers never cease to create new tools. In this research's case, a CPPo named DOK848 had demonstrated how this kind of chemicals will benefit mankind.

5.1 What is colchicine

Colchicine is an microtubule inhibitor extracted from *Colchicum autumnale* (which is also known as autumn crocus, meadow saffron or autumn lily) and other member of Colchiaceae family. Although in 6 century Alexander Trallianus described its usage to treat gout (Hartung, 1954), but only after French scientists Pelletier PS and Caventon J. extracted colchicine in 1820 (Pelletier PS and Caventon J, 1820), people began to realize that colchicine is the effective compound inside *Colchicum autumanle* against gout and familial Mediterranean fever (Surajana G, etc, 1997). In1937, Blakslee and Avery (Blakeslee A F and Avery A G, 1937) discovered that by introducing colchicine the chromosomes in plant cells would be doubled. People used to believe that colchicine’s effect on chromosomes comes from its ability to interfere the normal function and structure integrity of mitotic spindle (Eigsti O J and P. Dustin Jr, 1955), but the detailed mechanism was described in 1967 (Borisy G G and Taylor E W, 1967) and the binding site was identified as a protein had a sedimentation constant of 6S and suppose to be a subunit of microtubules.

The chemical structure of colchicine was elucidated by Dewar in 1945 (Dewar M J S, 1945). Colchicine has three rings, respectively A,B and C as shown in Figure 5.1. Among these rings, ring A is a analog of mescaline which could partially inhibit microtubule assembly, and ring C is a precise analog of tropolone methyl ether which could inhibit tubular polymerisation into microtubules in vitro (Andreu J M, etc, 1982). Studies shown that both the tropolone and trimethoxyphenyl site of colchicine could interact with tubulin (Andreu J M, etc, 1982 and Cortese F, etc, 1977). As for ring B, it plays in determining the binding speed and properties of colchicine to tubulin and affects colchicine-tubulin complex formation (Ray K, etc, 1981).

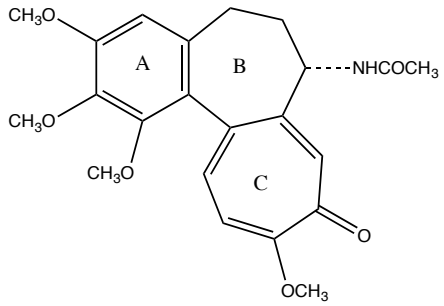


Figure 5.1 The structure of colchicine

Interestingly the working concentration of colchicine in plant cells and animal cells are hugely different. There are some theories about this phenomenon, such as amino acid substitution suggested by Banerjee etc in 2007. However no theory has been widely accepted by scientific community.

5.2 What is DOK 848

DOK 848 is a cell penetrating peptoid synthesised and provided by Porf. Dr. Schepers in ITG Karlsruhe Institute of Technology. Its chemical structure is shown in Figure 5.2. The chemical formula is $C_{130}H_{150}F_{44}N_{27}O_{25}$, and molecular weight is 3335.75. It contains three parts: a cell penetrating peptoid vector part, a colchicine cargo part and a rhodamine analog residue reporter part (see Figure 5.2). The linking parts of these two parts locates on the B ring of colchicine, so A ring and C ring are free to bind to tubulin (see Figure 5.1).

Due to the rhodamine analog residue, DOK848 could be traced inside cells under 588nm florescent light.

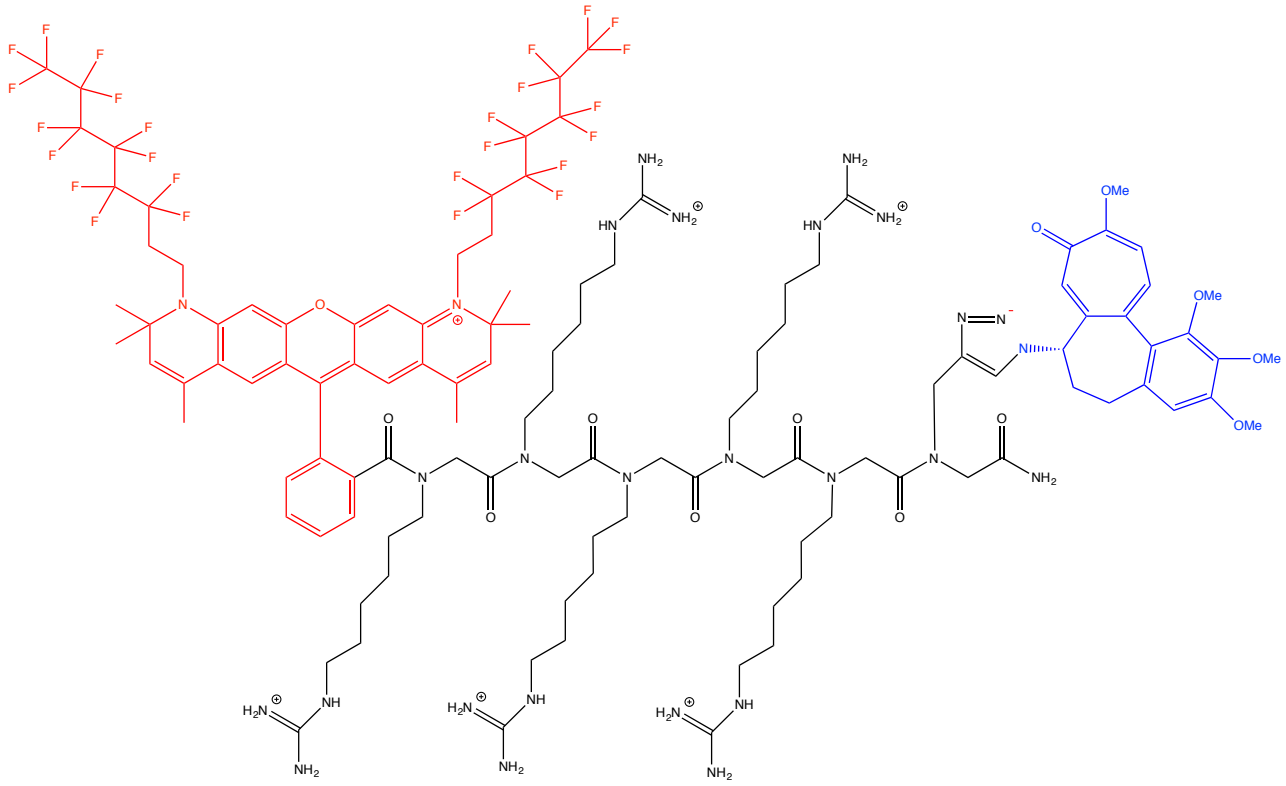


Figure 5.2 The structure of DOK 848: red part is rhodamine analog residue, black part cell penetrating peptoid vector part and blue part colchicine cargo part

As a CPPo has potential biological function, DOK848 should have these traits: 1. can enter cell with cargo molecule, in this case colchicine. 2. will not harm cell, or could keep cell in a relatively healthy status. 3. the cargo molecule could function normally in organisms. These traits will be examined in this study.

5.3 Assay Settings

To invested the function of DOK848 and compare the difference between the colchicine and DOK848, different concentration of colchicine and DOK848 have been used to treat Tub6 line.

5.3.1 Suspension cell culture and transgenic line

Suspension Transgenic BY-2 Tuβ6 cell line was used in this research. The details of Tuβ6 cell line culture is in 2.1.2.

5.3.2 Cell treatment

1mL BY-2 Tuβ6 cell have been transferred from erlenmeyer flask into 24 well plate. Certain amount of DOK848 and colchicine were added in due to different working concentration (see Table 5.1). Cells were shaking at 27 °C in the dark on horizontal shaker(KS250 basic, IKA Labortechnik, Staufen, Germany) at 200 rpm for 24 hours. Then . Control group setting were colchicine treated BY-2 Tuβ6 cells. Methods of treating and washing cell are as DOK848 treatment. Working concentration of colchicine also could be seen in table 5.1. For cell viability test, 1μM and 10 μM colchicine were also been used.

Chemicals	**Concentrations**		
Colchicine	100μM	150μM	200μM
DOK848	0.5μM	1.0μM	

Table 5.1 Concentration of colchicine and DOK848

From this table we could see that the working concentration of colchicine and DOK848 are hugely different in BY-2 cells

5.3.3 Image acquirement, process and analysis

All pictures were taken by spinning disc microscope AxioObserver Z1, as described in 2.6

Due to the nature of this assay, accurate signal intensity in the images are not required. Software Zen provided by Zeiss company (as described in 2.6)was used to increase the GFP signal intensity and reduce the background noises. Methods are described in 2.6.2.

5.4 Results

5.4.1 Phenotyping of treated BY-2 cell

Just like the CPPo library, the first two traits of DOK848 needed to be researched are: 1. the penetrating ability and 2. the toxicity to tobacco cells. Therefore, these two traits should be tested first.

For the penetrating ability of DOK848, 1μM DOK848 has been added to three days Tuβ6 cells. The cells were incubated in 24 well plate on horizontal shaker with 200 rpm for 24 hours, washed in the self-made washing chamber with sterile MS medium. Then BY-2 cells were then viewed under microscope. As it shows clearly in Figure 5.3, the microtubule in control cell remained contact while in treated cell have been disassembled., it means the DOK848 could penetrate through the cell membrane.

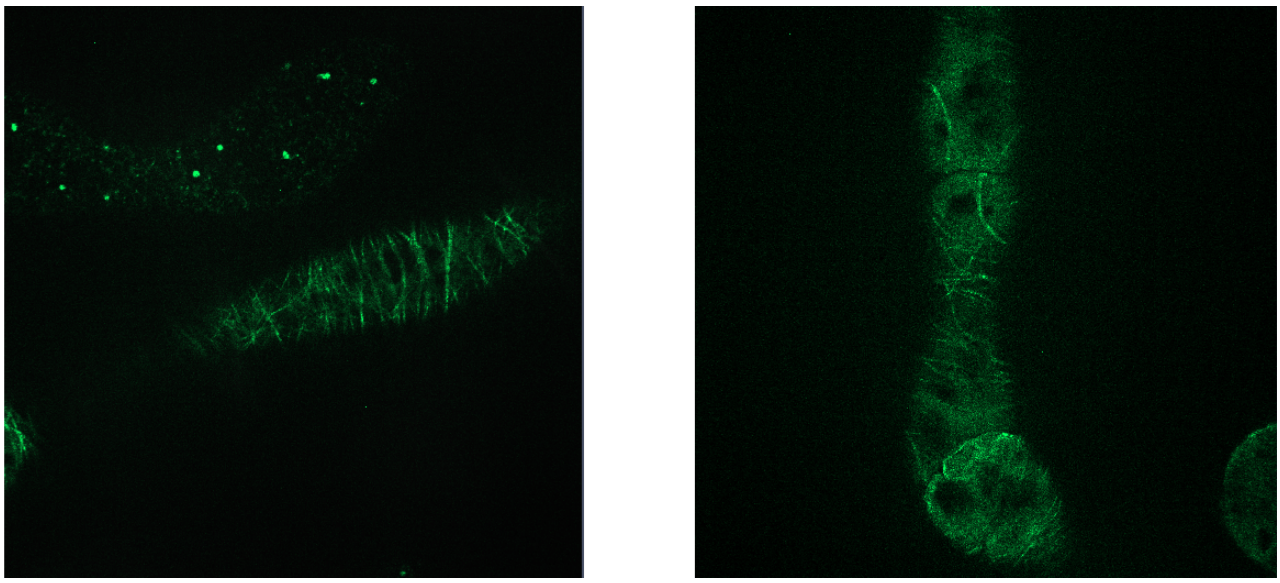
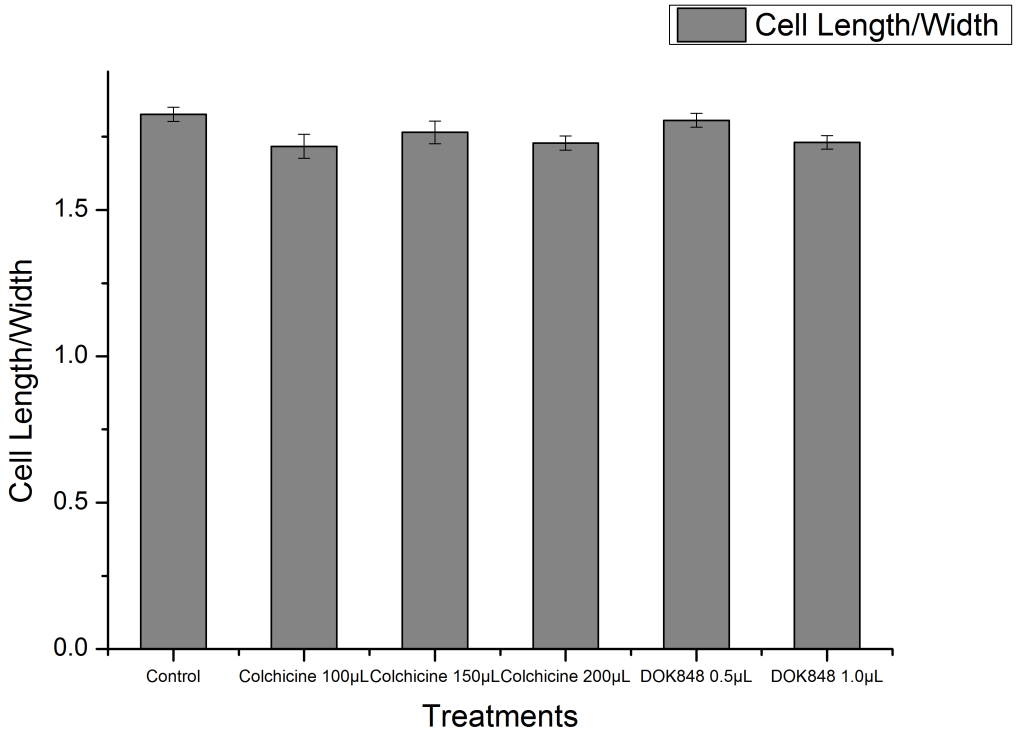


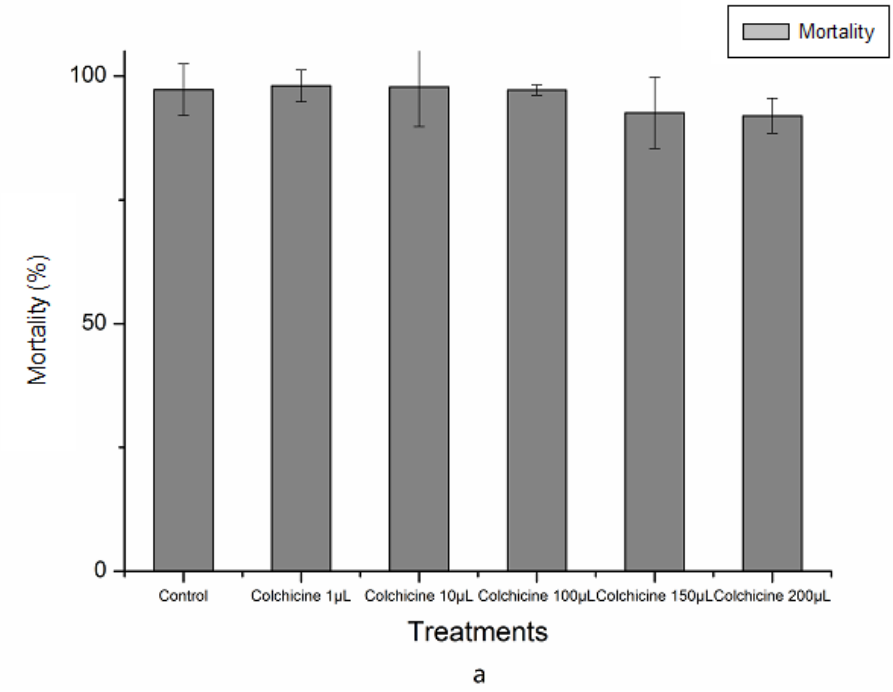
Figure 5.3 microtubule in 1μM DOK848 treated cell
The left picture is the control cell in which the cortical microtubule remained intact, the cell in the right picture is the DOK848 treated cell in which the cortical microtubule was disassembled

To investigate the toxicity of DOK848, two DOK848 treated Tuβ6 cells' phenotypes have been measured. The cell length/width ratio is measure using software imageJ, and this index could tell compare to WT cells, whether the treated cells are still in normal shape or not. The cell mortality is showing the percentage of surviving cells after treatment. As it is shown in Figure 5.4, the cell length/width ratio is lower down by the increasing of



concentration of colchicine and DOK848 comparing to control, but all the ratios are around 1.75. 1500 cells were measured in total.

Figure 5.4 The Cell Length/Width Ratio



The cell mortality of colchicine and DOK848 treated cells are shown in Figure 5.5, though the viability is lower by the increasing of the drug concentration as cell length/width ratio, however they are all above or around 90%. These two indexes shows that DOK848 is a safe vehicle that could bring colchicine into plant cells. Totally 1500 cells were observed.

on Example

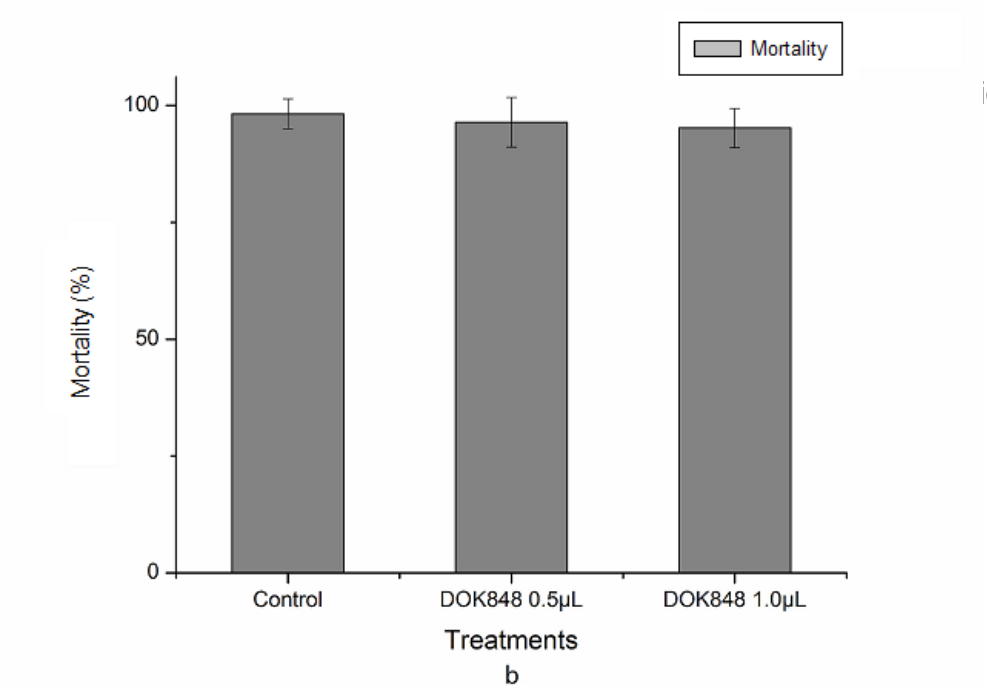


Figure 5.5 The Cell mortality of colchicine and Dok848 treated cells
Picture a is the mortality of colchicine treated cells and b the DOK848 treated cells

5.4.2 Microtubule inside BY-2 cell after treatment

To investigate the whether DOK848 have function inside BY-2 cells. Microtubule have been observed and measured after the treatment. Normally microtubule in BY-2 cells could be observed in two layers, cortical layer and radical layer. In cortical layer of untreated cells, normally microtubule would form a cylinder shape structure. From a optical section picture gained from confocal microscope, microtubule shows a clear structure of thin fine line that could be distinguished easily from one another. As it is shown in Figure 5.6, picture a shows the contact microtubule structure in control. a looks similar to picture d which is a picture of microtubule inside of a 100 μM colchicine treated cell. In pictures b and e, representing microtubule inside of 0.5 μM DOK848 and 150 μM colchicine treat cell respectively, dots become more than in a and d. These dots represent the disassembled microtubules inside cell, and this disassemble process was triggered by DOK848 in b and colchicine in e. In pictures c and f, microtubule were mostly disassembled, especially in c, there are nearly no contact microtubule left. However, by comparing the concentration of DOK848 and colchicine, a simple conclusion could be draw out clearly: with the help of peptoid part of DOK848, colchicine could penetrate into BY-2 cells and do its job in hundred fold lower concentration than it without any help.

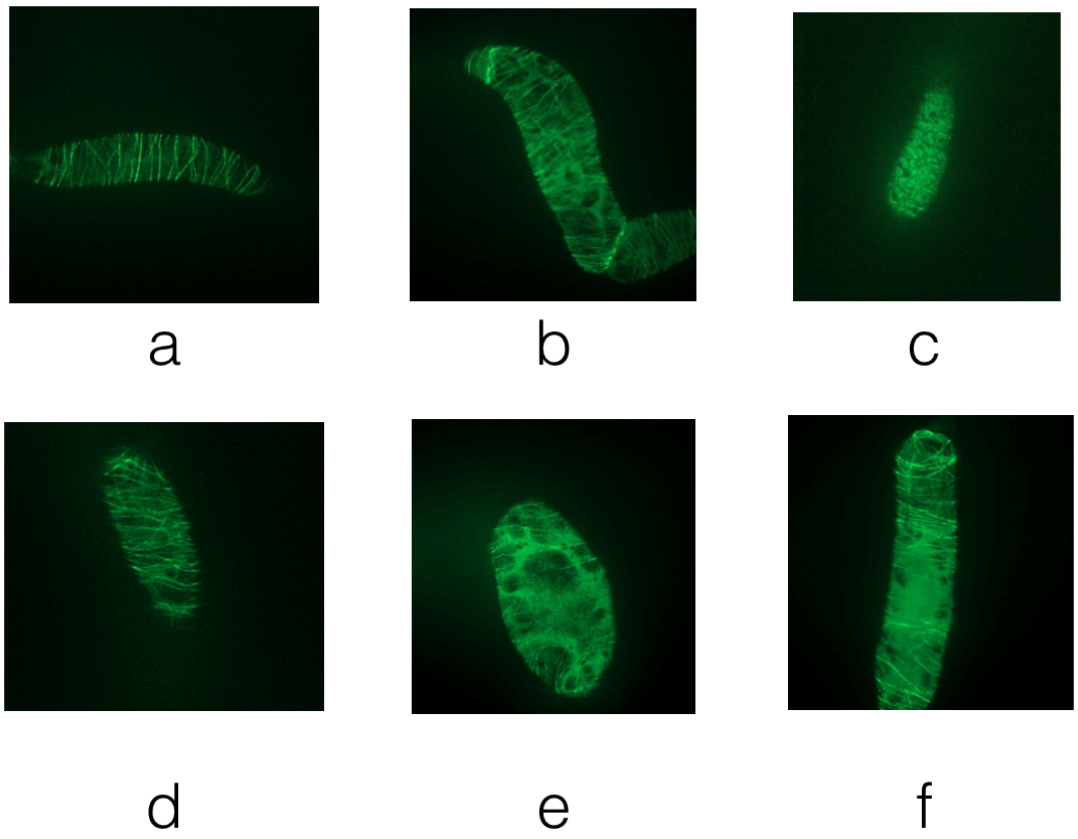


Figure 5.6 Colchicine and DOK848 treated Tuβ6 cells
a: control b: 0.5 μM DOK848 treated c: 1 μM DOK848 treated
d: 100 μM colchicine treated e: 150 μM colchicine treated f: 200 μM colchicine treated

5.5 Discussion

Though the penetration mechanism remains a mystery (just like nearly all the CPP and CPPo), DOK 848 shows its ability to carry a cargo molecule into plant cell, meanwhile the cargo molecule still can function normally (figure 5.6). Considering in application DOK848 may treat cells or plant for longer time, the incubation time extended from 2 hours to 24 hours, that make it possible for measuring more phenotyping indexes. As figure 5.4 and 5.5 b illustrate, DOK is also a non-toxicity drug to BY-2 cells comparing to CPPos in the library.

6 Outlook

Though only one library of CPPo and DOK 848 have been tested in this research, a bright light has shine in the future utilisation of this kind of molecule transporters. It can permeate into cells like CPP, but safe from peptide bond enzymes. A cargo molecule could be linked to a CPPo molecule after proper chemical design and synthesis, then it could be delivered into a target area and function at the time it should.

However for the research part, there is plenty left to be done.

1. What do the CPPo patterns mean?

   Every thing in nature has a reason, so do those patterns. For dot pattern, peroxisome theory could be examined. For membrane pattern, we still need to focus on receptor on membrane. Another approach is extend the incubation time, this could determine whether membrane pattern is just a pre-phase of other patterns. According to the time-series experiments result (figure 4.2), a membrane pattern like period occurs in the early stage of entry every patterns.

   For vacuole pattern, the mechanism of penetrating through vacuole membrane should be investigated.

2. Search for the mechanisms of CPPo entry

   From this research it only known that myosin plays role in CPPo distribution, but how are these CPPos penetrating in cells is still unknown. From the differences between penetrating speed, free diffusion is not possible. Endocytosis could be blocked by wortmannin, this may be a start.

3. Investigate the CPPo distribution patterns in root tips

   Patterns in root tips are interesting and have practical meaning in industry and agriculture. First, it could be find out that whether the patterns in suspension cells still exist in rice root tip cells. Second, could different patterns in rice root tip become engineering target?

   Science is no boundary and engineering always follow her steps, may the search never stop.

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