THE TRANSFORMATION OF GRAPE CALLUS WITH CHITINASE GENE

Krongjai Tasing

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2005

ISBN 974-533-437-5

การส่งถ่ายยีนไคติเนสเข้าสู่แคลลัสขององุ่น

ครองใจ ตะสิงห์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

มหาวิทยาลัยเทคโนโลยีสุรนารี

ปีการศึกษา 2548

ISBN 974-533-437-5

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ครองใจ ตะสิงห์ : การส่งถ่ายยีนไคติเนสเข้าสู่แคลลัสขององุ่น (THE TRANSFORMATION OF GRAPE CALLUS WITH CHITINASE GENE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. โชคชัย วนภู, 70 หน้า. ISBN 974-533-437-5

ปลายยอดขององุ่นสายพันธุ์ชีราสถูกเพาะเลี้ยงลงในอาหาร IM1, IM2 และ อาหารMM โดยเพิ่มความเข้มข้นของฮอร์โมน BAP (4.4 ไมโครโมลาร์, 8.8 ไมโครโมลาร์ และ 13.2 ไมโครโมลาร์ ตามลำดับ) พร้อมกับฮอร์โมน NAA ที่ความเข้มข้น 0.05 ไมโครโมลาร์ ยอดขององุ่นจำนวนมากถูกชักนำให้เกิดขึ้นภายใน 90 วัน ยอดและรากถูกชักนำโดยฮอร์โมน NAA ที่ความเข้มข้น 0.5 ไมโครโมลาร์ และ ฮอร์โมน Kinetin ที่ความเข้มข้น 0.9 ไมโครโมลาร์ ของอาหาร MS แล้วย้ายต้นองุ่นนี้ลงในกระถางเพื่อใช้สำหรับการขยายพันธุ์ต่อไป ยีนไคติเนสจากกระถินบ้านที่อยู่ในพลาสมิด pUC19 ถูกโคลนเข้าสู่เวคเตอร์ pET-39b(+) ตรงตำแหน่ง *Eco*RI จากนั้นยีนไคติเนสที่มีขนาด 1.1 กิโลเบส ถูกตัดที่ตำแหน่ง *SacI* และ *Bam*HI เพื่อโคลนเข้าสู่เวคเตอร์ pBI121 โดยนำเข้าไปแทนที่ยีน GUS จากนั้นจึงนำ pBI121: ไคติเนสนี้เข้าสู่ *Agrobacterium tumefaciens* สายพันธุ์ LBA4404 ด้วยวิธี Electroporation แล้วถ่ายโอนยีนนี้เข้าสู่องุ่นสายพันธุ์ชีราส โดยวิธี *Agrobacterium*-mediated transformation ใบองุ่นที่มี *Agrobacterium* ถูกเลี้ยงบนอาหารสูตร NN ที่มีฮอร์โมน 2,4-D ความเข้มข้น 5.0 ไมโครโมลาร์ และ ฮอร์โมน 4-CPPU ความเข้มข้น 5.0 ไมโครโมลาร์ นาน 2 วันในที่มืด แล้วจึงย้ายลงในอาหารสูตรเดียวกันนี้ที่มีสารปฏิชีวนะ ชนิด carbenicillin ความเข้มข้น 250 มก./ล. และ cefotaxime ความเข้มข้น 250 มก./ล. เพื่อกำจัด *Agrobacterium* จากนั้นคัดเลือกแคลลัสในอาหารสูตรเดิมแต่เพิ่มสารปฏิชีวนะชนิด kanamycin ความเข้มข้น 100 มก./ล. เข้ามาในอาหาร จากการทดสอบการถ่ายยีนด้วยการตรวจสอบยีนไคติเนสขนาดประมาณ 1.1 กิโลเบส และยีน NPTII ขนาด 0.8 กิโลเบส พบทั้งสองยีนนี้ในแคลลัสขององุ่นที่ถูกถ่ายยีน ชี้ให้เห็นว่าได้แคลลัสขององุ่นที่มียีนไคติเนสของกระถินบ้าน นอกจากนี้ เอนไซม์สกัดของกระถินบ้านสามารถทำลายสปอร์ของเชื้อรา *Plasmopara viticola* ได้สูงถึง 55%

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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____
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KRONGJAI TASING : THE TRANSFORMATION OF GRAPE CALLUS WITH CHITINASE GENE. THESIS ADVISOR : ASST. PROF. CHOKCHAI WANAPU. Ph.D. 70 PP. ISBN 974-533-437-5

CALLUS /CHITINASE/ GRAPE /TRANSFORMATION/TRANSGENIC GRAPE

Apical shoots of grape cultivar Shiraz were cultured on IM1, IM2 and MM medium with increased concentration of BAP (4.4 μM, 8.8 μM and 13.2 μM, respectively) and 0.05 μM NAA. The proliferated shoots were obtained in 90 days. The shoot and root were induced by 0.5 μM NAA and 0.9 μM Kinetin on MS medium and transferred into pots for propagation. The pUC19 contained chitinase gene of *Leucaena leucocephala* was transformed to pET-39b(+) vector at *Eco*RI site. About 1.1 kb of chitinase gene at *Sac*I and *Bam*HI sites were cut and replaced on GUS gene in pBI121. The electroporation method was used to transformed pBI121: chitinase to *Agrobacterium tumefaciens* strain LBA4404. This vector was transformed by *Agrobaterium* mediated transformation. Grape leaves were soaked with *Agrobacterium*, and put them on NN medium supplemented with 5.0 μM 2,4-D and 5.0 μM 4-CPPU for 2 days in dark and transferred to the same medium with 250 mg/l carbenicillin and 250 mg/l cefotaxime in order to eliminate *Agrobacterium*. The transgenic grape was selected on the same medium containing 100 mg/l kanamycin. The 1.1 kb of chitinase gene and 0.8 kp of NPTII selectable marker gene were found in transgenic callus. This indicated that leucaena chitinase was successfully introduced into grape callus. Furthermore, crude extract from *L. leucocephala* show high damage to *Plasmopara viticola* sporangia up to 55%.

School of Biotechnology
Academic Year 2005

Student's Signature Krongjai Tasing
Advisor's Signature
Co-advisor's Signature
Co-advisor's Signature

ACKNOWLEDGEMENTS

This research was successful, I would like to express acknowledgement my deep gratitude to all following;

Asst. Prof. Dr. Chokchai Wanapu, thesis advisor.

Prof. Dr. Nantakorn Boonkerd, co-advisor.

Dr. Sopone Wongkaew, co-advisor.

Asst. Prof. Dr. James Ketudat-Cairns, for the chitinase gene.

The National Science and Technology Development Agency (NSTDA), for financial support of this research.

All staffs and friends at F3 building of Suranaree University of Technology for all support in various things. Especially, Miss Sasidhorn Innok, Miss Ulaiwan Usansa and Miss Manunya Kongjaroen.

Most important, I would like to give the biggest thank to my parents, Mr. Juckrit Somrug for support, encourage and give the opportunity to studying in Graduated Program.

Krongjai Tasing

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LIST OF ABBREVIATIONS

2,4-D	=	2,4-Dichlorophenoxyacetic acid
4-CPPU	=	N-(2-Chloro-4-pyridyl)-N'-phenylurea
BAA	=	6-Benzyladenine
BAP	=	6-Benzylamipurine
bp	=	Base pair
BSA	=	Bovine Serum Albumin
IAA	=	Indoleacetic acid
IBA	=	Indolebutyric acid
IM	=	Initiation medium
kb	=	Kilobase
kDa	=	Kilo Dalton
kV	=	Kilo Volt
MB	=	Meristematic Bulk
MM	=	Maintain medium
MOP	=	(3-[N- Morpholino] propanesulfonic acid)
MS	=	Murashige and Skoog
NAA	=	Naphthalene acetic acid
NN	=	Nitsch and Nitsch
NOA	=	Naphthoxyacetic acid
NOS	=	Napaline synthase
NPTII	=	Neomycin phosphotransferase II

LIST OF ABBREVIATIONS (Continued)

OD	=	Optical Density
PCR	=	Polymerase Chain Reaction
rpm	=	Round per minute
SDS	=	Sodium dedocyl sulfate
TDZ	=	Thidiazuron
TSR	=	Template suppression reagent
µF	=	micro Farad

CHAPTER I

INTRODUCTION

1.1 Grapes

Grapes belong to the family Vitaceae, which include 14 living and two fossil genera and more than thousand species. The plants are herbaceous or woody vines with tendrils always arising opposite a leaf. Their inflorescences are generally located in place of a tendril but are rarely axillaries or pseudoaxillary. Plants are perfect or unisexual male or female (Pearson and Goheen, 1988).

1.2 Genus *Vitis*

Grapes are perennials, annually producing shoots that have tendrils. The more or less branched flower clusters are opposite the leaves. Wild plants are perfect (as are cultured grapevines) or unisexual male or female. Wild grapevine occurs primary in the Northern Hemisphere, especially in the temperate zone in Asia, North America, Central America, and Northwest South America in the Andes chain (Colombia and Venezuela). Cultivated grapevines now exist on five continents wherever the climatic conditions are favorable. In tropical and subtropical areas, grapevines may grow continuously and produce more than one crop per year.

The grapes are divided into two sections, *Vitis* (*Euvitis*) and *Muscadinia*. The section *Vitis* (*Euvitis*) consists of the grapevines. The canes have an ectracambia bark (including the pericyclic fibers and the primary and nonfunctioning secondary phloem) that may shed in strips. The secondary phloem has alternating tangential

layers of hard and soft phloem. A diaphragm interrupts the pith at the nodes. Berries ripen more or less evenly on the cluster. Seeds are general pear-shaped. The basic chromosome number is n = 19 or 2n = 38.

Asiatic species (*V. amurensis*, *V. davidii*, *V. armata*, *V. romanetii*, *V. piasezkii* and *V. coignetiae*, among others) are more or less susceptible to phylloxera and to the American grape diseases (black rot, downy mildew, and powdery mildew). The *V. amurensis* (which is native to the Amur River in Southern Siberia, Northern China, and Korea) has been used for breeding cold-tolerant hybrids. Some America species play important roles in genetic improvement of grapes around the world. Inter-specific America crosses have been used to obtain rootstocks resistant to a variety of soil pests and conditions. American species have been crossed with the European species, *V. vinifera*, to create both rootstocks and fruiting cultivars, including French-American wine grapes or French hybrids (also referred to as direct producers), that are more cold-hardy and phylloxera-resistant and less susceptible to fungus diseased than *V. vinifera* cultivar. The discussion that follows is limited to the important species of *Vitis* used in commercial viticulture, as scion cultivars or rootstocks, or in breeding programs.

***V. vinifera* L.**

The Old World species, *V. vinifera*, is the grape of antiquity often mentioned in the Bible. Most table, wine, and raisin grapes are produced from this variety. The *V. vinifera* originated in the regions between and south of the Caspian Black Seas in Asia Minor has been carried from region to region by civilized man in all temperate climates, and has been grown more recently in subtropical climates (Weaver, 1976).

The *V. vinifera* produces over 90% of the world's grapes, which are either pure *vinifera* or *vinifera* hybridized with one or more American species. About 85% of the grapes in the United States, grown mainly in California, are derived from pure *vinifera* varieties. The most important varieties of pure species in North America used for fruiting, with two varieties of each in parentheses, are the following: *V. labrusca* (Concord, Niagara*), V. rotundifolia* (Scuppernong, Eden), and *V. rupestris* (Rupestris St. George-used mainly as phylloxera-resistant rootstocks).

1.3 Structure and Growth stage of Vine

1.3.1 Structure of vine

The grapevine consists of two basic portions, the roots which are normally underground, and the trunk, arms, and shoots which are usually aboveground (Figure 1.1). The shoots consist mainly of stems, leaves, and flowers or fruits. The cells are the small structural compartments that make up tissues. Since they possess protoplasm (living material), they are the living units of vine structure and function. The vine consists of cells and the product of cells, and is an integrated collection of living and nonliving cells.

1.3.2 Growth stage of vine

Like most other plants, the grapevine has a fairly predictable cycle of growth. There are some grape growing areas in the world, such as the subtropical climates of southern India, where grape does not shed their leaves naturally. In the spring, when the mean daily temperature reaches about 10°C, the buds begin to swell spring, and the green shoots emerges form them. This is commonly known as bud

break. The shoots, leaves, tendrils, clusters, and new buds are rapidly grown and develop.

Blooming usually occurs around 8 weeks after bud break. The process of flower initiation for the following year's crop begins before bloom, and the development continues until harvest time (Figure 1.2).

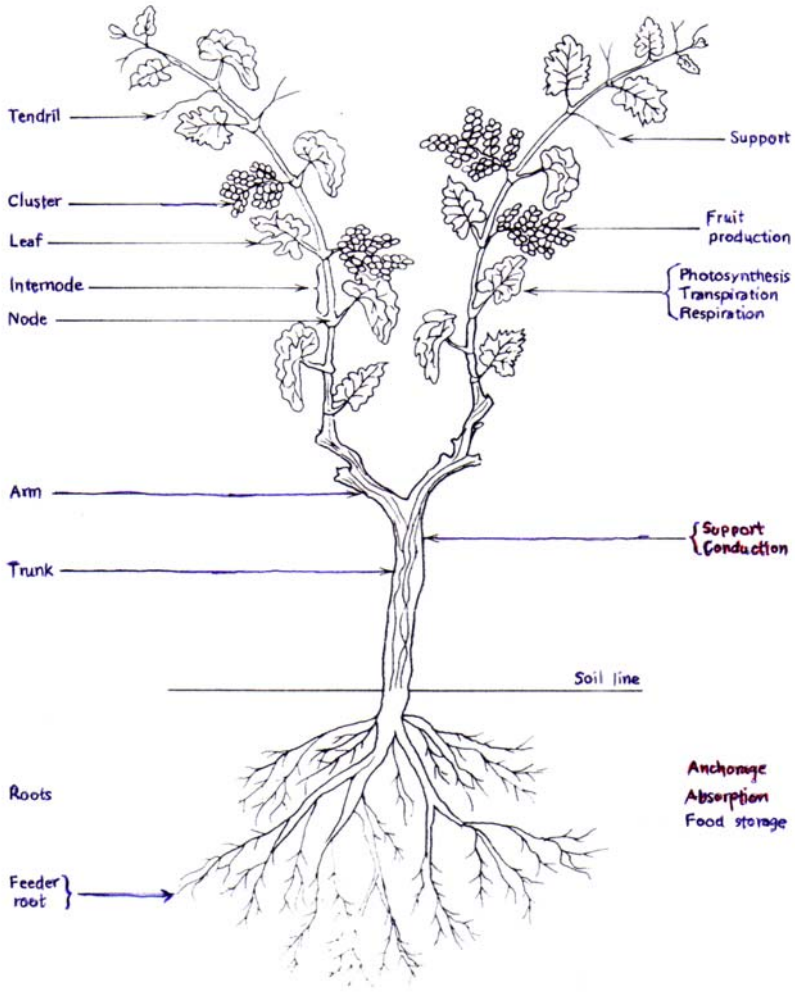


Figure 1.1 Diagrammatic illustration showing important structures and functions of a grapevine (Pearson and Goheen, 1988).

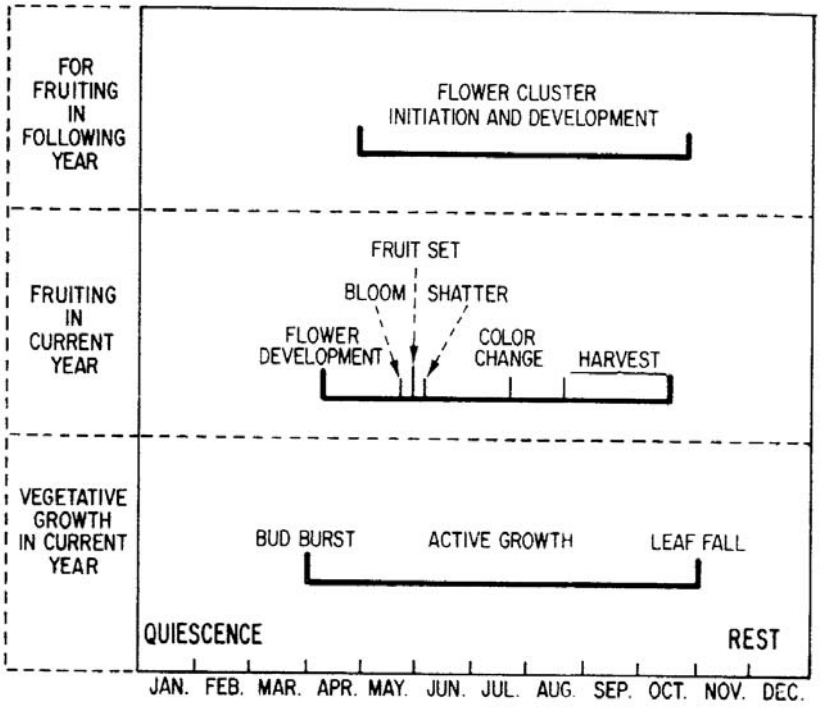


Figure 1.2 The stages in the growth and fruiting of *vinifera* grape in an average year in America (Pearson and Goheen, 1988).

1.4 Production of grape

The grape is a crop plant of many uses. Its fruit is fermented to wine and brandy. It is also eaten fresh. Grapes are divided into five main classes, depending on their purposes.

1.4.1 Table Varieties

These grapes are utilized for food and for decorative purposes. They must have an attractive appearance, good eating qualities, good shipping and storage qualities, and be resistant to injury incurred in handing.

1.4.2. Wine grapes

These varieties can produce satisfactory wine in some locations. The

majority of grapes grown throughout the world are utilized for wine making. For dry or table wines, grapes of high acidity and moderate sugar content are desirable, while grapes with high sugar content and moderately low acid are required for sweet or dessert wines. Variety grapes such as Shiraz, Carbernet Sauvignon, Riesling, and Pinot noir have the outstanding bouquet and flavor essential for production of highest quality premium wines.

1.4.3. Raisin grapes

These may include any dried grapes, although several standards must be met if a suitable dried raisin is to be made. The dried raisins must be soft in texture and should not stick together when storing. Few varieties can meet all of these criteria. Some of the best and most widely grown grape varieties for raisins are Thomson seedless, Black Corinth, and Muscat of Alexandria; the latter has seeds that can be removed by machine.

1.4.4. Juice Grapes

In the manufacture of sweet unfermented juice, the clarifying and preserving procedure should not destroy the natural flavor of the grape. In the United States grape juice is usually produced from Concord grapes or a blend of Concord and other varieties.

1.4.5. Canning Grapes

Only seedless grapes are suitable for use in canned fruit. The Thomson Seedless variety is most commonly used, alone or in combination with other fruits as fruit salad or fruit cocktail.

1.5 World vineyard acreage

The grape is the most widely planted fruit crop in the world, covering an area of approximately 10 million hectares (Pearson and Goheen, 1988). It grows from temperate to tropical regions, but most vineyards are planted in areas with temperate climates. In 2001 global vineyard acreage was 19.6 million acres. However, compared with the period 1997-2000 the most significant vineyard acreage increase in 2001 occurred in China (+57.9), Australia (+31.6%), New Zealand (+28.7%), Chile (+17.0%), United States (+9.7%) and Iran (+8.5%). But, some countries occurred decreases such as Azerbaijan (-74.9%), Lebanon (-25.5%), and Hungary (-20.6%). In 2001, Spain had the largest number of vineyard acres with 3,052,000 acres (15.6% of world total). France was second with 2,258,000 acres and Italy third with 2,244,000 acres. The Unites States had 971 acres of grape making it the fourth largest country in grape acreage (4.96% world grape acreage). This information is based on data collection of the International Office of Vine and Wine (OIV) located in Paris, France (Ivie International, 2003).

For Thailand, grape arrived during the period of King Rama the 5th of Rattanakosin, as reported by Department of Agriculture. In 1963, Pravin Poonnasee and his colleagues, Kasetsart University, Bangkok, tried to study and solve the problem of grape culture. The grape industry in Thailand has been a success since this time. At that time more than one hundred varieties were also introduced for testing from The United States and other countries. It was found that grapes could be adapted and grown well in Thailand. At present, grape has been grown cover many parts in Thailand, including in Suranaree University of Technology, Nakhon Ratchasima (Nantakorn Boonkerd, 2000).

In 1998, there are about 2,717 hectares producing 31,677 tones /year. The main area of grape production is located in the Central Plain area at Ratchaburi, Samut Sakhon, and Nakhon pathom, which mainly produce table grapes. In the Northeastern region, growers grew both table and wine grapes. There were two wineries at Loei and Nakhon Ratchasima province. In the Northern region, the table grape was grown in Chiang Mai and Nan province, while wine grapes were grown in Phichit province (Papademetriou and Dent, 2001).

1.6 Diseases of grape

Causal agents are fungi, bacteria, viruses, and nematodes. Disease in grape as in other crop plants, can results in substantial losses in production. In most cases, disease is the result of an interaction between a susceptible host and a living pathogenic organism. The effects of diseases on grape production are found throughout the record of viticulture. Diseases affect to production, harvesting, processing, marketing, and the consumer. The lower quality will be reduced yield, and increase the costs of producing and harvesting. Thailand is also similar to other countries. There are several diseases such as, anthracnose, powdery mildew including downy mildew which is a major disease of grapes in Thailand

1.6.1 Anthracnose: *Elsinoe ampelina*

Anthracnose is a disease of rainy, humid regions, where some grape cultivars are practically impossible to grow because of the disease. The optimal temperatures for disease development are 24-26°C. Conidia or ascospore formed on infected berries over-wintered on the vineyard floor may also cause primary infection. Anthracnose produces circular (1-5 mm in diameter) leaf lesions, with brown to black

margins and round or angular edges. The lesions are often quite numerous and may coalesce or remain isolated. The center of the lesions becomes grayish white and dries. The necrotic tissue eventually drops out of the lesion, leaving a “shot-hole” appearance. Young leaves are most susceptible to infection. Lesions may cover the entire blade or appear mainly along the veins. When the veins are affected, especially on young leaves, the lesions prevent normal development, resulting in malformation which is most obvious at the tips of the shoots, which appear burned. Young green succulent parts of the shoot are most susceptible to anthracnose. Lesions on shoots are small and isolated, with round or angular edges. Lesions have a violet-brown margin, which gradually become violet-black. Lesions may coalesce. The center of the lesions may extend to the pith of the shoot. Clusters are susceptible to infection before flowering and until veraison. Lesions on the rachis and pedicels appear similar to those on shoot (Pearson and Goheen, 1988).

1.6.2 Powdery mildew: *Uncinula necator*

The powdery mildew fungus can infect all green tissue of the grapevine. The fungus penetrates only the epidermal cells, sending haustoria into them to absorb nutrients. Although haustoria are found only in epidermal cells, neighboring noninvaded cells may become necrotic. The presence of mycelia with conidiaphores and conidia on the surface of the host tissue gives it a whitish gray, dusty or powdery appearance. Both surfaces of leaves of any age are susceptible to infection. Petioles and cluster stems are susceptible to infection throughout the growing season. Cluster infection before or shortly after bloom may results in poor fruit set and considerable crop loss.

U. necator may over winter as hypae inside dormant buds of the grapevine, as cleistothecia on the surface of the vein, or both. Developing buds are infected during the growing season. The fungus grows into the bud, where it remains in a dormant state on the inner bud scales until the following season. Shortly after bud break, the fungus is reactivated and covers the emergent shoot with white mycelium. Conidia are produced abundantly on these infected shoots and are readily disseminated by wind to neighboring vines. Rainfall causes disease by removing conidia and disruption of mycelium. Atmospheric moisture in the range of 40-100% relative humidity is sufficient for germination of conidia. Temperatures of 20-27°C are optimal for infection and disease development, although fungal growth can occur from 6 to 32°C (Pearson and Goheen, 1988).

1.6.3 Downy mildew: *Plasmopara viticola*

The downy mildews are primarily foliage blights that attack and spread rapidly in young tender green tissues, including leaves, twigs and fruits. Their development and severity in areas where are susceptible hosts and the respective downy mildew fungus is presented, depending greatly on the presence of a film of water on the plant tissues and on high relative humidity in the air during cool or warm, but not hot, periods. The reproduction and spread of the downy mildew fungi are rapid and their diseases show heavy losses in short periods of time. Downy mildew affects the leaves, fruit, and vines of grape plants and causes losses through killing of leaf tissue and defoliation, through production of low quality, unsightly or entirely destroyed grapes, and through weakening, dwarfing, and killing of young spring, when the mean daily temperature reaches about 10°C, the buds begin to swell

downy mildew can easily destroy 50-70 percent of the crop in one season.

Downy mildew of grape occurs in most parts of the world where it is warm and wet during the vegetative growth of the grapevine. (e.g. Europe, South Africa, Argentina, Brazil, Eastern North America, Eastern Australia, New Zealand, China and Japan). The European grape varieties are more susceptible than cultivated American grape type and the French hybrids. Among cultivars within *V. vinifera* are highly susceptible to downy mildew. *V. aestivalis* and *V. labrusca* are less susceptible, and *V. cordifolia, V. rapestris*, and *V. rotundifolia* are relatively resistant (Babadoost, 2001).

1.6.3.1 The pathogen

P. viticola is the lower fungi and details are listed below (figure 1.3)

Phylum: Oomycota which have elongated mycelium, produced zoospores in zoosporangia. Zoospores have two flagella. Sexual resting spores (oospores) produced by the union of morphologically different gametes.

Family: Peronosporaceae. Sporangium is borne by sporangiophores of determinate growth. This highly specialized family contains species that are all obligate parasites of vascular plants. The obligate parasites tend to remain between host cell, giving rise to specialized hyphal branches termed haustoria that extend through the host cell wall and invaginate the host cell plasma membrane.

Kingdom Chromista

Phylum Oomycota

Family Peronosporaceae

Genus *Plasmopara*

Species *viticola*

Figure 1.3 Taxonomy of *P. viticola*.

1.6.3.2 Symptoms

The pathogen attacks all green parts of vine, especially leaves. Leaf lesions appear as yellow or reddish brown areas on the upper leaf surface with corresponding white, downy or cottony fungal growth on the lower surface. Sometimes lesions are oily, somewhat angular and are located between the veins. Eventually all lesions become brown and dead with age. Severely infected leaves often fall prematurely. If enough defoliation occurs, the over wintering buds will be more susceptible to winter injury.

A. On leaves young infections are very small, greenish-yellow, translucent spots that are difficult to see. When the lesions enlarge, it appears on the upper leaf surface as irregular pale-yellow to greenish-yellow spots up to 1/4 inch or more in diameter (Figure 1.4). On the underside of the leaf, the fungus mycelium (the "downy mildew") can be seen within the border of the lesion as a delicate, dense, and white to grayish and cotton-like growth (Figure 1.5). Infected tissue gradually becomes dark brown, irregular, and brittle. Severely infected leaves eventually turn brown, wither, curl, and drop. The disease attacks older leaves in late summer and autumn, producing a mosaic of small, angular, yellow to red-brown spots on the upper surface. Lesions commonly form along veins, and the fungus sporulates in these areas on the lower leaf surface during periods of wet weather and high humidity (Ellis, www, 2004).

B. On fruit most infection occurs during 2 distinct periods in the growing season. The first is when berries are about the size of small peas. When infected at this stage, young berries turn light brown and soft, shatter easily, and under humid conditions are often covered with the downy-like growth of the fungus

(Figure1.6). Generally, little infection occurs during hot summer months. As nights become cooler in late summer or early fall, the second infection period may develop. Berries infected at this time generally do not turn soft or become covered with the downy growth. Instead, they turn dull green, then dark brown to brownish-purple. They may wrinkle and shatter easily and, in severe cases, the entire fruit cluster may rot. These infected fruit will never mature normally.

Figure 1.4 Downy mildew on upper side of grape leaf (Ellis and Nita, www, 2005).

Figure 1.5 Appearances of infection on under side of grape leaf (Ellis and Nita, www, 2005).

Figure 1.6 Downy mildew on young grape berries (Ellis, www, 2004).

C. On shoots and tendrils early symptoms appear as water-soaked, shiny depressions on which the dense downy mildew growth appears (Figure 1.7). Young shoots usually are stunted and become thickened and distorted. Severely, infected shoots and tendrils usually die.

Figure 1.7 Downy mildew symptoms on shoot (Babadoost, 2001).

1.6.3.3 Causal organism

P. viticola, the mycelium diameter varies from 1 to 60 μm because

the hyphae take up the shape of the intercellular spaces of the infected tissues. The mycelium grows between the cell but sends numerous, globose haustoria into the cell. In humid weather, the mycelium produces sporangiophores, which emerge on the underside of the leaves and on the stem through the stomata or rarely by pushing directly through the epidermis. In the young fruit, sporangiophores emerge through lenticels. Usually 4-6 sporangiophores arise through a single stomata, but sometimes there may be as many as 20. Each produces 4-6 branches at nearly right angles to the main stem of the sporangiophore, and each branch produces 2, sometimes 3, secondary branches in a similar manner. At the tips of the secondary branches, single, lemon-shaped sporangia (conidia) are produced (Figure 1.8). The sporangia are blown by the wind or are transported by water, and in the presence of free moister they germinate. Sporangia generally germinate by means of zoospores, which emerge from the sporangia, swim about for a few minutes, encyst, and then produce a germ tube by which they can infect the plant. In rare instances sporangia may germinate by directly producing a germ tube. It can be classified into 2 reproductions, depending on environment.

A. Asexual reproduction occurs by the formation of sporangia, which are ellipsoid and hyaline and measure 14x11 μm. Sporangia are borne on treelike sporangiophores (140-250 μm long) (Figure 1.9). Each sporangium gives rise to one to 10 biflagellate zoospores measuring 6-8x4-5 μm. The zoospores escape from the side of the sporangium opposite its point of attachment, either through an opening in a papilla or by directly perforating the wall. Zoospores are mainly uninucleate. Protoplasmic fusion between hyphae originating from different zoospores may occur inside parasitized tissues and give rise to heterokaryotic mycelium (Pearson and Goheen, 1988).

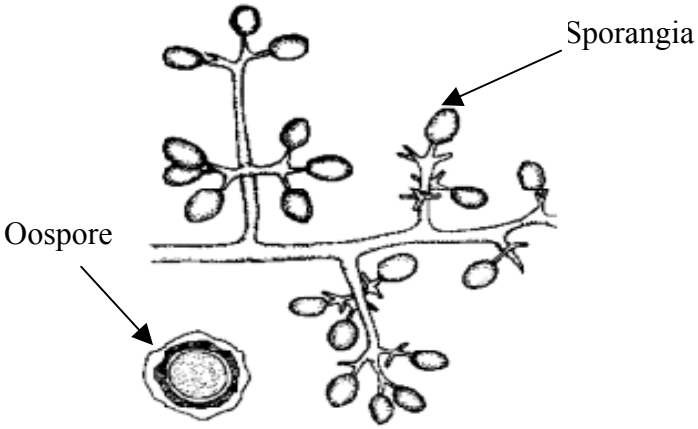


Figure 1.8 *P. viticola*, the grape downy mildew pathogen, seen under high-power microscope. Thick-walled oospore (lower left). Branched sporangiophores bearing terminal, lemon-shaped sporangia (Babadoost, 2001).

Figure 1.9 Sporangia of *P. viticola* on treelike sporangiophores emerge through stomata on the under side of leaf (Pearson and Goheen, 1988).

B. Sexual reproduction begins early in the summer and occurs by the fusion of an antheridium and an oogonium derived from the terminal expansion of hyphae (seen in figure 1.10). An oospore (20-120 μm in diameter) forms and is enveloped by two membranes and covered by the wrinkled wall of the oogonium.

Oospores from in leaves or possibly throughout the parasitize organ. The following spring, oospores germinate in free water, giving rise to one or occasionally two slender germ tubes, 2-3 μm in diameter and of variable length. The germ tubes terminate in a pyriform sporangium (28x36 μm), which produce 30-56 zoospores (Pearson and Goheen, 1988).

1.6.3.4 Disease cycle

P. viticola overwinters mainly as oospores in fallen leaves, although it can survive as mycelium in buds and in persistent leaves, the latter in regions with mid winters (Figure 1.10). Oospores survive best in the surface layers of moist soil, survival is little affected by temperature. Oospores germinate in water in spring as soon as temperatures reach 11°C to produce a sporangium from which primary dispersal of zoospores.

Sporangiophores and sporangia are produced only through the stomata of infected organs. It requires 95-100% relative humidity and at least 4 hours of darkness. The optimal temperature for sporulation is 18-22°C. The sporangia are detached from sporangiophores by dissolution of a cross wall of callose, moisture again being required. The sporangia are dispersed by wind to leaves, where they germinate in free water (optimal temperature 22-25°C) to release zoospores.

Zoospores swim and encyst near stomata, which are entered by germ tubes from germinating cysts. Because the fungus penetrates the host exclusively via the stomata, only those plant structures with functional stomata are susceptible to infection. Under optimal conditions, the time from germination to

penetration is less than 90 minutes. Downy mildew is favored by all factors that increase the moisture content of soil, air, and host plant. Therefore, rain is the principal factor promoting epidemics. Temperature plays a less important role by retarding or accelerating for development of the disease. The optimal temperature for development of the fungus is about 25°C, the extremes ranging from 10 to 30°C.

The most serious epidemics of downy mildew occur when a wet winter is followed by a wet spring and a warm summer with intermittent rainstorms every 8-15 days. These conditions also spread cover the vineyard in spring. Successive periods of rain stimulate the production of young, susceptible shoots with functional stomata (preparatory rains) and ensure their infection (infection rains).

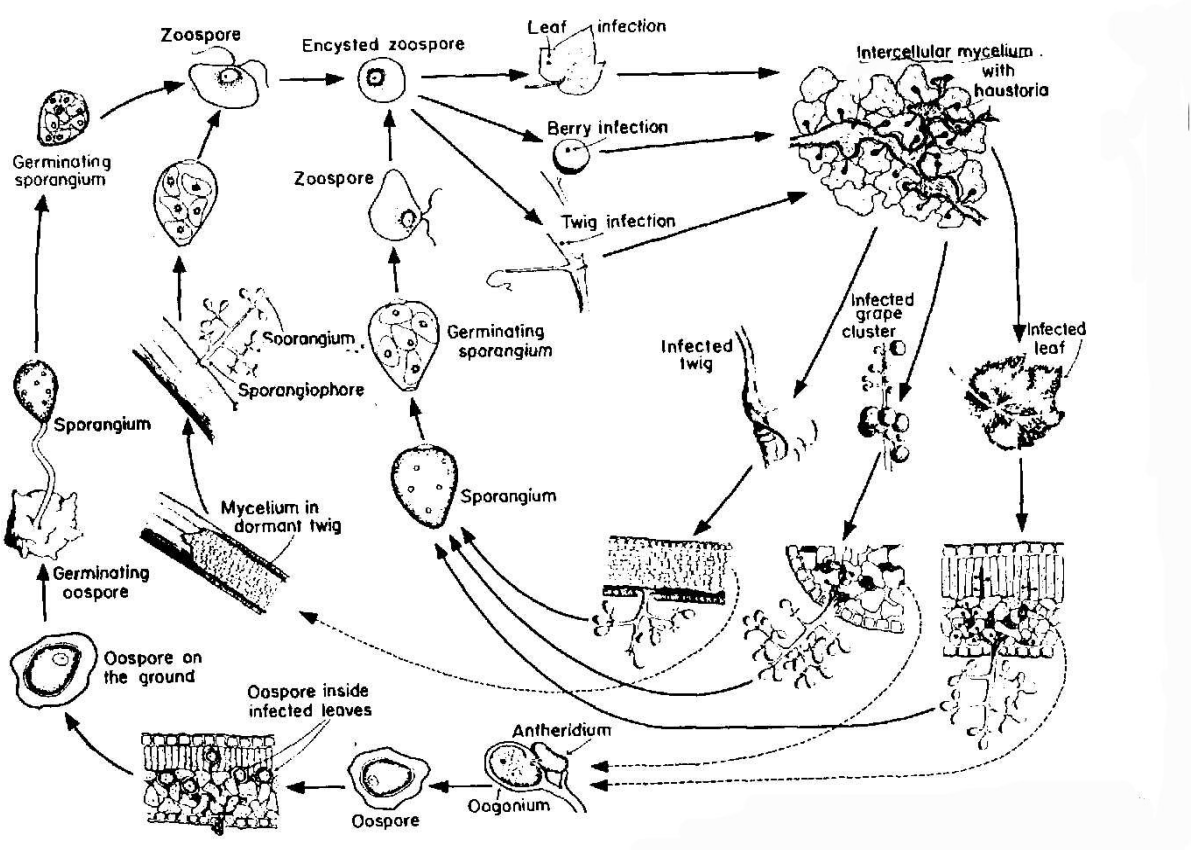


Figure 1.10 Disease cycle of *P. viticola* (Balasubramaniam, Harvey, Braithwaite, and Jordan, www, 2005).

1.7 The enzyme: chitinases

Chitinases (EC 3.2.1.14) are enzyme that catalyze the hydrolysis of β-1,4 linkage in chitin, a linear homopolymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc) residue (Figure 1.11) and belong to the group of pathogenesis related proteins (PR-Proteins). Chitinases are presented either constitutively or after the induction in plant. They accumulate coordinately in plants during pathogen attack and response to UV light, mechanical wounding, chemical stress, ethylene treatment, etc. Chitinases can be found in plants, fungi, bacteria, insects, fishes and protozoa (Henrissat, 1991 quoted in Datta and Muthukrishman, 1999). Plants respond to attack by pathogenic microorganisms by the induction of expression of the large number of genes encoding diverse proteins, many of which are believed to have a role in defense. Based on sequence comparison, chitinases have been subdivided into six classes (Figure 1.12). These classes can be grouped into two families of glycosyl hydrolasase, family 18 and family 19 (Henrissat, 1991 quoted in Datta and Muthukrishman, 1999). Chitinases from classes I, II, and IV are of plant origin and make up the family 19 glycosyl hydrolases. These chitinases share homologous catalytic domain in addition to the signal peptide found in all of them. Family 18 chitinases have also been classified as classIII, V, and VI which have been found in a wide range of species, including bacteria, fungi, plants, insects, mammals, and viruses.

Class I chitinases The prototypes of this class are the tobacco basic chitinase. They have an N-terminal cysteine-rich chitin binding domain (CBD), a glycine and proline-rich region (hinge region), and a highly conserved catalytic domain. This chitinases are synthesized as precursor with an N-terminal signal sequence,

which direct them to the secretary pathway.

Class II chitinases The chitinases of tobacco, petunia and *Arabidopsis* were identified as class II. They lack a CBD and the proline-rich regions, but have a high amino acid sequence homology to the main structure of class I chitinases.

Class III chitinases They are found in cucumber, *Heaver* and *Arabidopsis*; they have no sequence similarities to class I and class II chitinases, but have high sequence identity with chitinases from yeast and zygomycetes. They lack the Cysteine-rich domain and hinge region.

Class IV chitinases They are found in sugar beet, rape, and bean which contain a cysteine-rich domain and a conserved main structure but differ from class I chitinases in having deletion in the catalytic domain and being smaller in size.

Class V chitinases This class is represented so far by a single protein. When the cDNA for the precursor of stinging nettle (*Urtica dioica*) lectin was cloned, it was a big surprise to discover that the precursor was synthesized as a chitinase homology with two CBDs (Datta and Muthukrishman, 1999). A duplicated N-terminal lectin domain and an amino acid sequence, which are similar to the stinging nettle lectin precursor. Chitinase are structurally unrelated to classes I, II and IV.

Class VI chitinases The only class VI chitinase known so far was isolated from sugar beet. They have no sequence similarity to class I-V chitinases but have

a significant amino acid sequence similarity to bacterial exochitinases. The individual chitinases from the same species often differ in their physio-chemical and biological properties. In general conclusion, some class I chitinase is localized in vacuoles, whereas other chitinases are located apoplatically (Collinge et al., 1993).

The molecular weight of most plant chitinase is about 30 kDa (Hahn, Schlersier, and Hohne, 2000). Chitinases of 40-90 kDa and some as high as 120 kDa have been obtained from arthropods and some vertebrates such as fish, amphibians and mammals. A wide range of molecular size from 30 to 120 kDa is observed in bacteria and fungi. Chitinases have the following wide range or pI values: 3.0-10.0 in higher plants and algae: 4.7-9.3 in insects, crustaceans, mollusks and fishes; and 3.5-8.8 in microorganism. Therefore, acidic and basic chitin are presented in these organisms. The optimum pH of chitinases are 4-9 for higher plants and algae, pH 4.8-7.5 for animals and pH 3.5-8.0 for microorganisms. (Jolles and Muzzarelli, 1999).

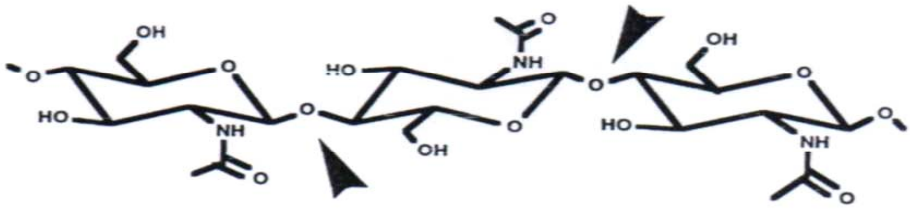


Figure 1.11 The structure of chitin and the position chitin's cleaved by chitinase (arrow) (Datta and Muthukrishman, 1999).

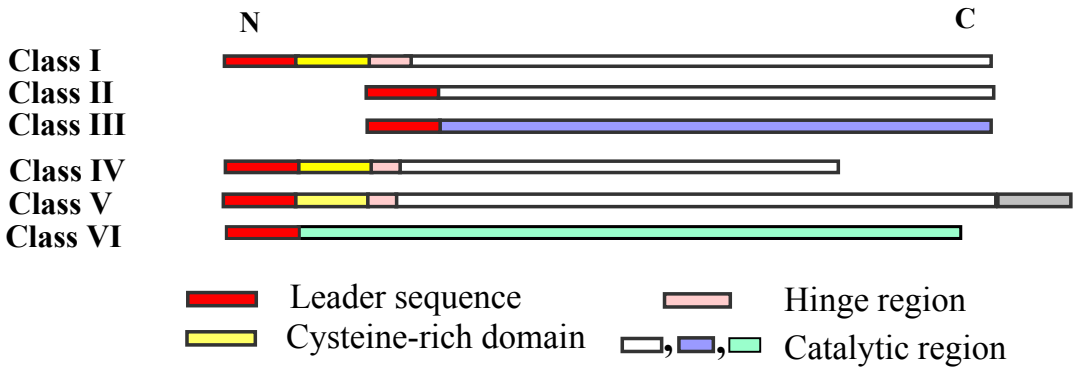


Figure 1.12 Model of chitinase classes.

Plant chitinase such as *Leucaena leucocephala* de Wit have a molecular weight of 46 kDa and the isoelectric point of 7.5. It has a pH optimum of 4.5 and temperature optimum of 55°C (Mana Kaomek, Poonsuk Sriyotha, Mizuo, Fujimura, and Ketudat Cairns, 2003).

There are many research in plant defend mechanism. Chitinase is main focus of this study. The character of chitinase is to degrade the chitin which is a major component of pathogens cell walls. Natural chitin is major component of the exoskeleton of insects and other arthropods, and the cell wall of most fungus. Fungi have 3-60% of chitin in cell wall (Bartnicki-Farci, 1968; Wessels and Sietsma, 1981 Quoted in Mana Kaomek, 2001). Chitin is linear β-1,4 linkage polymer of N-acetylglucosamine (GlcNAC), and one of the most abundant polysaccharides in nature (Henrissat, 1991 quoted in Datta and Muthukrishman, 1999).

Chitinase has been purified from plants such as barley, bean, cabbage, corn, potato, rice, rye, soybean, tobacco, wheat, grapevine and yam. Verburg and Huynh (1991) showed *Arabidopsis* chitinase is an effective inhibitor of growth of

Trichoderma reesei, as assessed by the hyphal extension assay. Huynh et al. (1992) showed that Maize chitinase could inhibit the growth of *Fusarium oxysporum* (potato wilt), *Alternaria solani* (tomato early blight). Also, Ponstein et al. (1993) used a microtiter dish assay to determine the effects of the purified enzyme on the growth *in vitro* of *F. solani*. Proteins were added to a suspension of pregerminated spores on a layer of potato dextrose agar. After an incubation period of 2 hours, the germling suspensions were microscopically monitored for lysis. Therefore, chitinase from tobacco was capable of causing lysis of *F. solani* germlings. Leo et al. (1994) displayed the antifungal activity of chitinase by testing the lysis activity of this protein *in vitro*. Chitinase caused lysis and complete growth inhibition of *T. viride* at a concentration of 2 μg per well. On the phytopathogenic fungus *A. radicina,* chitinase significantly inhibited fungal growth at 5-10 μg per well. These results from previous studies supported the hypothesis that the *in vivo* role of these pathogenesis-related proteins can protect the host from invasion by fungal pathogens. Mana Kaomek et al. (2003) demonstrated that to test chitinase from *L. leucocephala* could inhibit growth of 13 fungi.

1.8 Plant tissue culture and transgenic plant

One of the main objectives of plant transformation is to develop plant tissue culture for callus, multiple shoot, and plantlet inducing. For induction the explants to cultivate the callus, multiple shoot, and plantlet, various kinds of media (inorganic salt media) have been designed. Agar or its substitutes are added into the media to prepare solid media for inducing. One of the most commonly used media for plant tissue culture is that developed by Murashige and Skoog (MS) for plant tissue culture.

Component of media for the growth of plant tissue culture can be classified into six groups. There are Major inorganic nutrients, trace element, iron source, organic supplement (vitamins), carbon source (sucrose), and organic supplements (plant growth and regulations).

In 1991, Marchenko studied embryogenesis in primary from leaves and stem of grape plants. Callus formed after 20-60 days and transferred to media containing 1 mg/l 2,4-D and 2 mg/l BAP (modified MS media). In addition, Vilaplana and Mullins (1989) showed that adventitious buds were formed on hypocotyls and cotyledons when mature dormant somatic embryos of *V. vinifera* L. cultivars Sultana (syn. Thomson Seedless) and Grenache and Gloryvine were cultured on Nitsch and Nitsch (NN) medium with 10 μM BAP. Buds arising on hypocotyls gave rise to shoots and subsequently to plants. Emershad and Ramming (1994) demonstrated that somatic embryo formation which were occurred from immature zygotic embryos within of seedless grape (*V. vinifera* L.) by culturation for two months on liquid Emershas/Ramming medium. Somatic embryos continued to proliferate after excision and transfer to Emershas/Ramming medium supplemented with 1 μM BAP and 0.65% TC agar. Plant development occurred on Woody plant medium supplemented with 1.5% sucrose, 1 μM BAP, 0.3% activated charcoal and 0.65% TC agar.

In 1995, Harst reported that an efficient regeneration protocol was developed for leaf explants derived from *in vitro* plantlet of grapevine cultivar Seyval blanc. High frequency somatic embryo induction could be obtained on a modified NN69 medium with 20 μM NOA in combination with the synthetic cytokinin TDZ (4 μM). Embryos could be induced on almost 80% of the plated leaf discs, and the regeneration capacity could be maintained for at least two years. Interestingly,

Salunkhe, Rao, and Mhatre (1997) shown that somatic embryogenesis was observed in callus initiated from tendril explants of *V. vinifera* L. cultivars Thomson, Sonaka and Tas-e-Ganesh on Emershad and Ramming medium supplemented with 1 μM BAP. Emerging shoots subsequently formed completely plantlets on liquid rooting medium containing 1 μM IAA. However, Jayasnkar, Gray, and Litz (1999) demonstrated that embryogenic suspensions of grapevine (*V. vinifera* L.) which were initiated from somatic embryos of 'Thomson Seedless' and 'Chardonnay'. Suspension cultures consisted of proembryonic masses (PEM) that proliferated without differentiation in a medium containing 2, 4-D. Somatic embryos were matured and regenerated into plant in MS basal medium containing 3% sucrose. It was found that more than 60% of the somatic embryos were regenerated to plantlet. Also, Salunkhe et al. (1999) reported that anthers of *V. latifolia* L. (wide grape) were cultured on Nitsch and Nitsch medium supplemented with 20 μM 2,4-D and 9 μM BAP to produce callus after 4-6 weeks. Callus was subcultured on Nitsch and Nitsch medium contain 10 μM NAA to produced somatic embryos within 6 weeks. Then, on the somatic embryos which grew on regulator-free Nitsch and Nitsch basal medium were converted to plantlets in 6-8 weeks. Mhatre and Salunkhe (2000) indicated that nodal explants bearing a single axillary bud, from three cultivars of cultivated grape, cultivars 'Thomson Seedless', 'Sonaka' and 'Tas-e-Ganesh' were used for initiate shoot cultures on G16 medium containing adenine sulphate, monobasic sodium phosphate, BAP and NAA. Each shoot was developed from an axillary bud, produced tuft of multishoots on a medium containing BAP, calcium pantothenate, monobasic sodium phosphate and IBA. Subculture of the tuft of multiple shoots to an elongation medium resulted in distinct individual shoots. Rooting of shoots and plantlet

formation was achieved on IAA- containing liquid medium. While, Singh, Khawale, and Sigh (2004) illustrated that nodal segments of grape cultivars Pusa Urvashi and Pusa Navrang were cultured on MS medium supplemented with 2.0 or 4.0 mg/l BAP, 0.2 mg/l BAA. The sprouted shoots were successfully proliferated and rooted on half-strength MS medium containing 2.0 mg/l IBA, 200 mg/l activated charcoal. The elongated shoots in each subculture could be used for multiplication on the same medium after 6-7 weeks, by excising them into two-node micro-cutting. The rooted plantlet (after three weeks of inoculation) which obtained the above medium was successfully hardened in glass jars filled with peat: soilrite (1:1).

Therefore, there were many researches about chitinases in transgenic plants that can reduce the damage caused by pathogens such as the work of Nakano, Hoshino, and Mii (1994), Scorza, Cordts, Ramming, and Emershad (1995), Kikkert, Wallance, Striem, Reisch, and Hebert-soule (1996), Kikkert, Wallance, Striem, Reisch, Ali et al. (1997), Tabei et al. (1998), and Yang, Ingelbrecht, Lonzada, Skaria, and Mirkov (2000). The rice chitinase gene, classified as class I chitinase, was introduced into the somatic embryos of grapevine (*V. vinifera* L. cultivar Neo Muscat) by *Agrobacterium* infection. Two treatments showed enhanced resistance against powdery mildew caused by *U. necator*. Fewer disease symptoms were observed on leaves of these transformants compared with those of the non-transformant, although browning and necrotic symptoms, which seemed to constitute a hypersensitive reaction were observed. Scanning electron microscopic observation revealed that conidial germination, mycerial growth and conidial formation were suppressed on the leaf surface of the transformed grape. The transgenic grapevines obtained also exhibition slight resistance against *E. ampelina* causing anthracnose, resulting in a

reduction in diseased lesions (Yamamoto et al., 2000). Neverless, Harst, Bornhoff, Zyprain, and Topper (2000) reported that somatic embryos originating form anther cultures of *V. vinifer* cultivars Dornfelder, Muller-Thurgau and Reisling which cultured on solid or in liquid medium were used for transformation via *Agrobacterium tumefaciens* strain LBA4404. After two days of co-cultivation the embryos proliferated were cultured in liquid NN69 medium containing kanamycin for selection and cefotaxime for removal of bacteria. Subsequently cultivation of larger embryos was carried out on solid NN69 medium supplemented with kanamycin. Highest conversion rates of selected germinated embryos as rooted plantlets were obtained from embryos of suspension cultures.

The endochitinase gene from *T. harzianum* strain P-1 was successfully introduced into “Merlot” and “Chardonnay” grapevines by biolistic transformation of embryogenic cultures (Kikkert, Ali, Wallance, and Riesch, 2000). The gene was expressed at high levels in some of the transgenic lines and in preliminary tests appeared to have activity against *Botrytis cinerea* and *U. necator*. Mezzetti, Pandolfini, Navacci, and Landi (2002) reported that transgenic grape plant (Thomson Seedless and Silcora) were carried out a method based on regeneration via organogenesis. *In vitro* proliferating shoots were cultured in the presence of increasing concentration of BAP. After three months, produced meristematic bulk tissue was characterized by a strong capacity to differentiate adventitious shoots. A slice which was prepared from the meristematic bulk was used for *Agrobacterium*-mediated transformation of grape plants with the DefH9-iaaM gene. Grape plants were transferred to the field.

In addition, other plants have been a transgenic plant such as cucumber,

peanut, tobacco, soybean and potato. Tabei et al. (1998) reported that a rice chitinase cDNA (RCC2) was introduced into cucumber through *Agrobacterium* mediation. More than 200 putative transgenic shoots were regenerated and grown on MS medium supplemented with 100 mg/l kanamycin. Sixty elongated shoots were examined for the presence of the integrated RCC2 gene and subsequently confirmed to have it. Of these, 20 independent shoots were tested for resistance against gray mold (*B. cinerea*) by infection with the conidia: 15 strains out of 20 independent shoots were exhibited a higher resistance than the control (non-transgenic plants). Furthermore, Rohini and Rao (2001) showed that the expression of the heterogous chitinase gene driven by CaMV 35s promoter led to a high level of activity in some of the transgenic plants. Small-scale field tests indicated that these plants were increased ability to resist the the fungal pathogen *Cercospora arachidicola* (the causal organism of the leaf spot or Tikka disease of peanut), which is an important peanut pathogen.

Therefore, the purpose of this research is to obtain a grape callus transforming with chitinase gene. The scope and limitations of the study will be as following; (1) grape plants will be regenerated by tissue culture method, (2) the damage ability of *L. leucocephala* chitinase to *P. viticola* sporangia will be evaluated, (3) the chitinase gene into expression vector will be cloned, (4) transgenic grape callus will be obtained.

Objectives

1. To obtain the grape plants by tissue culture method
2. To evaluate the damage ability of *Leuceanu leucocephala* chitinase to *Plasmoara viticola* sporangia
3. To transform a chitinase gene into pET-39b(+), expression vector (pBI121) and grape callus
4. To detect the foreign gene (NPII and chitinase gene) in transgenic grape callus

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plants

Grape plants (*Vitis vinifera,* cultivars Shiraz, Loose Perlet, Crimson Seedless, Carolina Black Rose, Ruby red, and Reisling) were collected from vineyard of Suranaree University of Technology (SUT) Farm, Nakhon Ratchasima, and used for tissue culture, chitinase activity assay and transformed with chitinase gene.

Leucaena leucocephala (*L. leucocephala*) were collected from SUT, Nakhon Ratchasima. Protein of *L. leucocephala* shoots were extracted for chitinase activity assay, and *P. viticola* sporangia were evaluated damage ability of chitinase.

2.1.2 *P. viticola* sporangia

Initial sporangia of *P. viticola* were collected from a lesion of grapevine leave from SUT Farm, Nakhon Ratchasima. The sporangia were propagated and detached grapevine leaves under controlled condition (explained in 2.2.2.5). The sporangia were used for evaluation the lysing efficacy of chitinase enzyme from *L. leucocephala*.

2.1.3 Cloning and expression vector and host cells

The chitinase gene was integrated in pUC19 vector (shown in Appendix) and obtained from Asst. Prof. Dr. James Ketudat-Cairns, SUT. The pET-39b(+) plasmid

vector (shown in Appendix) was obtained from Novagen (Madison, W1, USA). *Escherichia coli* strain DH5 was generally used as a host cell for amplification of recombinant plasmid. *A. tumefacients* strain LBA4404 was used for transformation of chitinase gene into grape callus, and a binary vector pBI121 was used as expression vector (shown in Appendix).

2.1.4 Equipments

The equipments were used as following: PCR model GeneAmp PCR system 2400 and 9700 of PERKIN ELMER, Sequencer model ABI Prism 310 Genetic Analyser, Gel electroporasis of Toyobo model Gel mate 2000, Gel electrophoresis of Bio-rad model Power PAC 300. UV-transmittance of Ultra-Violet Products model White/Ultraviolet Transilluminator, Electroporation model Gene Pulser II of Bio-rad, Biological incubator, Centrifuge of Heraeus instrument model Labofuge 400R, model SORVAL RC5C PLUS, Centrifuge of eppendorf model Centrifuge 5415c. Water bath of Scientific promotion Ltd. model Maxi-shake, Comport Heto master shake, Growth chamber model Contherm, Hot air oven, Refrigerator (4°C), Freezer (-20°C and -70°C), Laminar flow hood, pH meter, Compound microscope and basic microbiological, plant tissue culture and molecular equipments.

2.1.5 PCR Primers

The oligonucleotides were obtained from Bio Basic Inc. (Canada). The chitinase and NPTII primers were designed for detection the foreign gene of transgenic plants as shown in Table 2.1.

2.1.6 Chemicals and molecular reagents

Chemicals (analytical grade and molecular grade) were purchased form Fluka Chemika, Across, Sigma, Bio-Basic, Promega, and Phyto Technology Laboratories including, T_4 DNA ligase, restriction endonuclease (*Bam*HI, *Eco*RI, *Nde*I, and *Sac*I), Taq polymerase, Oligonucleotides primer, QIA Prep Spin Miniprep kit (Germany), and antibiotics.

Table 2.1 Oligonucleotides were designed from iPCR website (Bordoli, Falquet, and Ioannidis, www, 2004). Chitinase forward is part of CaMV35s promoter and chitinase reverse is part of NOS terminator.

Primers name	Oligonucleotides sequence	Tm (°C)	GC (%)
Chitinase forward	5' ACGTAAGGGATGACGCACAA3'	61.3	50
Chitinase reverse	5' CAAATGTTTGAACGATCGGG3'	61.1	45
NPTII forward	5' CCATAAATTCCCCTCGGTATCC3'	62.6	50
NPTII reverse	5' CCGCTCAGAAGAACTCGTCAA3'	62.8	52.4

2.2 Methods

2.2.1 Grape tissue culture

The apical shoot of grape cultivar Shiraz was surface sterilized in 70% ethanol for 10 seconds and was soaked in 1% sodium hypochlorite solution with Tween 20 for 10 minutes. The samples were rinsed 3 times in sterile water for 10, 5

and 5 minutes, respectively. Explants were transferred to the required solid medium and hormone supplement to induce proliferate shoots and meristematic bulk (MB). The medium were used for the initiation (IM) of the MB containing KNO_3 (1050 mg/l), NH_4NO_3 (400 mg/l), KH_2PO_4 (200 mg/l), $MgSO_4 \cdot 7H_2O$ (400 mg/l), $CaNO_3$ (750 mg/l), NaH_2PO_4 (200 mg/l), microelements and vitamins by MS, 3% sucrose; pH 5.8 and 0.7% commercial agar supplemented with 0.05 µM NAA (Mezzetti et al., 2002). IM medium was supplemented with 4.4 µM BAP for the first 30 days subculture, after which the BAP concentration was doubled (8.8 µM) for the second 30 days of subculture. MB was maintained on IM medium supplemented with 13.2 µM BAP (MM medium). The proliferate shoots were cut in a small slice by using sterile scalpel to transfer the shoots to fresh medium for shoot and root induction (the medium was MS, pH 5.8 supplemented with 0.5 µM NAA, 0.9 µM Kinetin, 1.5% agar and 3% sucrose). The plantlets were transferred into pots for propagation.

2.2.2 Evaluation the damage ability of crude extract from *L. leucocephala* to *P. viticola* sporangia

2.2.2.1 Extraction of crude extract from *L. leucocephala* shoots

Five grams of *L. leucocephala* shoots were ground in liquid nitrogen using a cold mortar and transferred to a centrifuge tube (30 ml) with cold 0.01 M sodium acetate buffer, pH 5.0. The extracts were centrifuged at 13,000 rpm at 4°C for 30 minutes. The supernatants (crude extract) were collected for chitinase assay, protein determination, and taken for chitinase damage ability evaluation.

2.2.2.2 Extraction of crude extract from grapes

One gram of shoots, stem-internodes and leaves of 7 cultivars grapes (Loose perlet, Crimson Seedless, Carolina Black Rose, Ruby Red, Shiraz, Reisling, and Root Stock cultivar 1613) were ground in liquid nitrogen using a cold mortar and transferred to a centrifuge tube (30 ml) with cold 0.1 M sodium acetate buffer, pH 5.0. The extracts were centrifuged at 13,000 rpm at 4°C for 15 minutes. The supernatants (crude extract) were collected for chitinase assay and protein determination.

2.2.2.3 Chitinase activity assay

Chitinase assay was modified from Krishnaveni, Liang, Muthukrishman, and Manichkarm (1999) using colorimetric assay with Carboxymethyl-Chitin-Remazol Brilliant Violet (CM-Chitin-RBV) as substrate (1 mg of chitin azure (Sigma) suspended in McIlvane's buffer, pH 5.5, in a total volume of 1 ml McIlvane's Buffer: 82 ml of 0.2 M sodium phosphate dibasic (Na_2HPO_4), 18 ml of 0.1 M citric acid). A 400 µl of sample was incubated at 40°C for 10 minutes with 0.2 ml of 0.1 M sodium acetate buffer, pH 5.0 and 0.2 ml of CM-Chitin-RBV substrate. The reaction was terminated by adding 0.2 ml of 2 N HCl and cooled on ice for 10 minutes. Then, the samples were centrifuged at 13,000 rpm for 5 minutes at 4°C. The absorbance of the supernatants was determined at 550 nm.

2.2.2.4 Protein content determination

Protein concentration was determined by Bradford method. A 20 µl of sample was added in 1.0 ml Bradford reagent and incubated at room temperature for 5 minutes. Then, the reactions were measured at 590 nm and the

BSA protein was a protein to constructed standard curve.

2.2.2.5 Evaluation the damage ability of crude extract to *P. viticola* sporangia

The sporangia of *P. viticola* were collected from grape leaves of a lesion. The sporangia suspension was sprayed on grape leaves in box (shown in Appendix) under 25°C, 98% humidity, in 8 hours /16 hours dark/illumination of growth chamber for 7 days. Then, 300 µl of sporangia suspension ($4.15x10^4$ sporangia/ml) was mixed with 0.011, 0.021, and 0.042 unit (Unit = Optical density at 550 nm of Remazol Brilliant Violet released per minute) of crude extract from *L. leucocephala* in a centrifuge tube and incubated at room temperature for 3 days. The normal number of sporangia were counted daily under a microscope for 3 replications and compared with sample treated with buffer (control). Number of normal sporangia/ml = Normal sporangia x 2,000 (Tuite, 1969) and percentage of damaged sporangia was calculated for evaluation the damage ability of chitinase.

2.2.3 Transformation of chitinase gene into pET-39b(+), pBI121 and grape callus

2.2.3.1 Cloning of chitinase gene into pET-39b(+) vector

A. Preparation of plasmid DNA

E.coli of chitinase encoding gene in pUC19 plasmid and pET-39b(+) plasmid vector were cultured in LB medium containing amplicilin and kanamycin, respectively. Culture was incubated overnight at 37°C with vigorous shaking. Plasmid DNA was isolated from small-scale (1-2 ml) bacterial cultures by treatment with alkaline and sodium dedocyl sulfate (SDS) (Sambrook and Russel, 2001). The cells of

E.coli were collected at 13,000 rpm for 1 minute and resuspended in 100 µl of ice cold alkaline lysis solution I (50 mM glucose, 25 mM Tris-Cl, pH 8.0, and 10 mM EDTA, pH 8.0), followed by added 250 µl of denaturating solution (0.2 N NaOH, and 1% SDS). The mixture was gently mixed by inverting the tube 4-6 times. Then, 150 µl of cold solution III (5 mM potassium acetate and glacial acetic acid) was added and the tube was immediately inverted for 4-6 times and incubated on ice for 3-5 minutes. The DNA solution was separated by centrifugation at 14,000 rpm for 5 minutes, and transferred the supernatant to a fresh tube. An equal volume of phenol:chloroform (25:25) was added and centrifuged at maximum speed for 2 minutes at 4°C. The aqueous upper layer was transferred to a fresh tube for precipitation of DNA. The nucleic acid was precipitated by adding 2 volumes of absolute ethanol at room temperature and centrifuged at maximum speed for 5 minutes at 4°C. The pellet was washed by adding 1 ml of 70% ethanol and centrifuged at maximum speed for 2 minutes at 4°C. The nucleic acid was dissolved in 50 µl of TE (pH 8.0). As a result, DNA was screened by agarose gel electrophoresis and stored at –20°C.

B. Chitinase digestion and gel purify fragment

The DNA solution was placed in a sterile microcentrifuge tube and was mixed with *Eco*RI and buffer. The reaction mixture (60 µl) was composed of 40 µl of pUC19 plasmid (4.97 µg/µl), 3 µl (10 unit/µl) *Eco*RI, 6 µl of 10xNE buffer, 0.6 µl of 100xBSA and 10.4 µl distilled water, incubated at 37°C for 3 hours. The result of DNA digesting was screened by agarose gel electrophoresis. Then, QIAGEN quick Kit was used for purification of chitinase gene. The DNA fragment was excised from

the agarose gel with a clean sharp scalpel. Then, the gel was weighted and dissolved in 3 volumes of QG buffer incubated at 50°C for 10 minutes. Then, 1 volume of isopropanol was added to the sample and mixed. A QIAquick spin column was placed in a 2 ml provided collection tube in order to bind DNA. The QIAquick column was centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded and placed QIAquick column in the same collection tube. A 0.75 ml of PE buffer was added and centrifuged twice for 1 minute at 13,000 rpm. The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube for elution DNA by adding 50 µl of EB buffer (10 mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane and centrifuged the column for 1 minute. The agarose gel electrophoresis was done for screening size of chitinase gene. The DNA solution was stored at –20°C.

C. Preparation of DNA vector

Isolation of plasmid DNA pET-39b(+) was followed by Sambrook and Russel, 2001. DNA was digested with *Eco*RI and incubated at 37°C for 3 hours as described in part A and B of 2.2.3.1. The result was determined by agarose gel electrophoresis and the DNA solution was stored at –20°C.

D. Preparation of competent cell

The competent cells of DH5α *E.coli* were prepared by modified $RbCl_2$ method of Hanahan, (1968) Quoted in Sambrook,and Russel (2001). A single colony of *E. coli* was inoculated to 2 ml of LB broth and incubated at 37°C with vigorous shaking for overnight. Then, 1 ml of cell culture was inoculated into 50 ml of LB broth and incubated the culture until the optical density (OD) at 600 nm was

approximately 0.5. The cells were transferred into sterile ice cold 50 ml tube, chilled on ice for 15 minutes and recovered them by centrifugation at 4,500 rpm for 5 minutes at 4°C. The supernatant was pour off from the cell and pellet was resuspended in 5 ml of chilled TFBI solution (30 mM potassium acetate, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, and 15% v/v glycerol) and put on ice for 10 minutes. The pellet was centrifuged in the same condition and supernatant was decanted. The cells were resuspended in 500 µl of chilled TFBII solution (10 mM MOPS, 75 mM $CaCl_2 \cdot 2H_2O$, 10 mM $RbCl_2$ and 15% v/v glycerol) extremely gently each pellet. A 100 µl aliquots of competent cells were transferred to sterile microcentrifuge tubes and kept at -70°C.

E. Ligation of DNA fragment into vector and transformation

The vector of DNA was heated at 65°C for 5 minutes, then put immediately on ice for 2 minutes and transferred to a sterile microcentrifuge tube. The purified chitinase gene fragment, T_4 DNA ligase and ligation buffer was mixed together. The reaction mixture (15 µl) was composed of 1 µl of pET-39b(+) vector (2.212 µg/µl), 11 µl of eluted DNA fragment (1.365 µg/µl), 1 µl of T_4 DNA ligase, 1.5 µl of 10xligation buffer, and 0.5 µl of ddH_2O. The reactions mixture was incubated at 16°C, overnight. Then, 100 µl of competent cells were thawed on ice for 5 minutes. Ligation mixtures were added to competent cells gently mixed by swirling and stored on ice for 30 minutes. The cells were heat shocked at 42°C for 90 seconds and the tube was rapidly transferred on ice for 2 minutes. The transformed cells were grown by adding 800 µl of LB medium and incubated the culture at 37°C for 40 minutes. Then, 200 µl of the culture was spreaded on LB plate containing 50 µg/µl of

kanamycin. The plate was incubated at 37°C, overnight. The recombinant clones were detected on LB plate supplemented with kanamycin. Then the result was checked by *Eco*RI and *Nde*I digestion on agarose gel electrophoresis.

2.2.3.2 Cloning of chitinase gene into pBI121 expression vector

Chitinase gene was integrated into pET-39b(+) plasmid at *EcoR*I site. The plasmid was prepared by alkaline lysis with SDS method (Sambrook and Russel, 2001). Then, DNA was digested by *Bam*HI and *Sac*I for preparation of vector and chitinase gene fragment. The reaction mixture (60 µl) was consisted of 40 µl of binary vector pBI121 (1.6 µg/µl), 2 µl (10 unit/µl) *Bam*HI, 2 µl of *Sac*I, 6 µl of 10x multiple core buffer, 0.6 µl of 100x BSA and 9.4 µl distilled water. The mixture was incubated at 37°C for 3 hours. The results of DNA digest was screened by agarose gel electrophoresis. The pET-39b(+): chitinase was digested and purified by QIAGEN quick Kit. The purified chitinase gene fragment, T_4 DNA ligase and ligation buffer were added together. The reaction mixture (10 µl) was contained 2 µl of pBI121 vector (1.6 µg /µl), 5.4 µl of eluted DNA fragment (0.14 µg /µl), 1 µl of T_4 DNA ligase and 1 µl of 10xligation buffer and 0.6 µl of ddH_2O. The reactions mixture was incubated at 14°C, overnight. A 100 µl of competent cell was thawed on ice for 5 minutes. The ligation mixtures were added to competent cell, gently mixed by swirling and the tube was stored on ice for 30 minutes. The cells were heat shocked at 42°C for 90 seconds and the tube was rapidly transferred on ice for 2 minutes. The transformed cells were grown by adding 800 µl of LB medium and incubated the culture at 37°C for 1 hour. Then, 300 µl of the culture was spreaded on LB plate containing 50 µg/µl of kanamycin, and was incubated at 37°C, overnight. The

recombinant clones were detected on LB plate supplemented with kanamycin and checking the results by *Bam*HI and *Sac*I digestion on agarose gel electrophoresis.

2.2.3.3 Sequencing of pBI121: chitinase using chitinase forward primer

The *E.coli* of chitinase encoding gene in pBI121 was cultured in LB medium containing 50 µg/µl of kanamycin on vigorous shaking at 37°C, overnight. The plasmid DNA was isolated by QIA Prep Spin Miniprep kit (Germany). About 193 ng (7.72 µl) of DNA was used for template, 2 µl of terminator ready reaction mix, 1 µl of 5x big dye buffer and 1.28 µl (3.2 pmol) of chitinase forward primer (shown in Table 2.1) were mixed together in PCR tube. Then, the reactions were placed to GeneAmp PCR system 9700. The PCR condition was conducted as followed, 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. After the PCR experiment was finished, extension reactions were placed into the tube of sodium acetate/ethanol mixture (3.0 µl of 3 M sodium acetate, pH5.0, 62.5 µl of 95% ethanol and 14.5 µl of ddH_2O) and mixed thoroughly. The tube were vortex and leave at room temperature for 15 minutes to precipitate the extension products. Then, the solutions were centrifuged at 13,000 rpm for 20 minutes. Supernatant was removed from the tube by using pipette tip. The pellet was rinsed with 250 µl of 70% ethanol and centrifuged for 5 minutes at 13,000 rpm. The supernatant was discarded and dried the tube with the lid open in a heat block at 90°C for 1 minute. A 15 µl of TSR was added to each sample pellet and mixed thoroughly on a vortex mixer and then, heated for 2 minutes at 95°C. Immediately, the sample tube was chilled on ice and transferred to a new 0.5 ml sample tubes and cover with tube septa for running on the ABI PRISM 310 Genetic Analyzer.

2.2.3.4 Introduction of pBI121: chitinase gene into *A. tumefaciences* LBA4404

A pBI121: chitinase was constructed by substituting the GUS gene in pBI121 vector, which was introduced into *Agrobacterium* by electroporation method.

A. Preparing electro-competent cell

A single colony of *Agrobacterium* was inoculated to 3 ml of LB broth containing 50 µg /ml kanamycin and 20 µg /ml rifampicin. Then, it was shaken in incubator of 28°C for overnight. A 1.5 ml of an overnight culture was added into 50 ml of LB containing appropriate antibiotics and incubated the culture until the optical density at 600 nm as approximately 0.5. The culture was transferred into chilled 250 ml centrifuge tube and centrifuged at 4°C for 10 minutes at 4,500 rpm. The pellet was resuspended in 50 ml of ice cold sterile ddH_2O, centrifuged. This step was repeated again. Then, the pellet was repeated in 100 ml of ice cold sterile ddH_2O and centrifuged at 4°C for 15 minutes at 4,500 rpm and resuspended in 100 ml of ice cold 10% glycerol and centrifuged at 4°C for 15 minutes at 4,500 rpm. Finally, 500 µl of 10% ice cold glycerol was added into 80 µl electro-competent cell of each 1.5 ml centrifuge tubes and placed in -70°C refrigerators.

B. Introducing binary plasmid into *Agrobacterium* by electroporation

An 80 µl of electro-competent cell was taken form -70°C and thawed on ice. A 1 µl (about 3.32 µg) of binary vector was added into the electro-competent cell and mixed together. Then, the suspension was transferred into a cold electroporation cuvette (1 mm electrode gap). The electroporation was carried out by using 2500 V, 25 µF, 50Ω, 1.3 ml/second impulse condition and 900 µl of LB medium was added

into the cuvette, quickly resuspended the cell. Then, the suspension was transferred to 1.5 ml centrifuge tube and incubated in shaker incubator (200 rpm) at 28°C for 2 hours. A 200 µl of each transformation was pipette on LB plate with 50 mg/l kanamycin and 20 mg/l rifampicin. The cells were spreaded with bent glass rod and the plates were placed inverted at 28°C for 2-3 days. The transformed colonies were observed by the plasmid mini-preparation checking for the insert through restriction digestion (*Bam*HI and *Sac*I).

2.2.3.5 Transformation of chitinase gene into grape callus

Transforming *Agrobacterium* was cultured in the LB medium broth containing 50 µg/ml kanamycin and 20 µg/ml rifampicin at 28°C (180 rpm) overnight. Then, the bacterial culture was transferred to 50 ml LB medium containing the same antibiotics and incubated until the cell was approximately 0.5 at 600 nm. The bacterial cells were centrifuged at 13,000 rpm for 2 minutes at 4°C. The pellets were resuspended in 200 ml NN medium supplemented with 1 mM acetosyringone.

Leaves from plantlets were surface sterilized in 70% ethanol for 10 seconds and was soaked in 1% sodium hypochlorite solution with Tween 20 for 10 minutes. The samples were rinsed 3 times in sterile water for 10, 5 and 5 minutes, respectively. Then, Leaves were cut into pieces of 0.5 cm square. These leaves were soaked with *A. tumefaciences* suspension and incubated for 10 minutes. After infection, the pieces were blotted with sterile filter papers and placed on NN medium supplemented with 5 µM 2,4-D, 5 µM 4-CPPU, 0.8 % agar and 3% sucrose at pH 5.8. After 48 hours in the dark at 28°C, the leaf explants were washed with water, treated with 250 mg/l carbenicillin and 250 mg/l cefotaxime for 5 minutes to kill

Agrobacterium, and then transferred to the same medium enriched with 250 mg/l carbenicillin and 250 mg/l cefotaxime for 7 days. The cuttings were transferred to the selective medium supplemented with 250 mg/l carbenicillin, 250 mg/l cefotaxime and 100 mg/l kanamycin and maintained for 30 days. These explants were cultured under a 16 hours/light, 8 hours/dark, in a clean room, which was controlled at 25°C then, transferred to fresh selective medium every 4-5 weeks.

2.2.4 Detection of foreign gene in a transgenic grape callus

Genomic DNA was isolated from callus by a DNA mini-preparation method (Lin, Ding, Li, and Kuang, 2001). The callus (25-100 mg) was placed into a 1.5 centrifuge tube. The tube was caped and thrown into liquid nitrogen for 10 seconds. A 200 µl pipette tip was used for callus homogenizing into a fine powder immediately. The obtained tissue powder was suspended in 600 µl of DNA extraction buffer (100 mM Tris-Cl (pH 8.0), 50 mM EDTA (pH8.0), 500 mM NaCl, 2% SDS (w/v), 2% β-mercaptoethanol (v/v), and 1% PVP (w/v)) and mixed thoroughly. Then, the tube was incubated at 65°C in a water bath for 15 minutes. The tube was centrifuged at 12,000 rpm for 10 minutes at 4°C and transferred the supernatant to a new tube. A 10 µl of RNase A was added and incubated at 37°C for 10 minutes. The suspension was purified twice using phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 12,000 rpm for 3 minutes. The DNA was precipitated by adding 0.6 volume of ice-cold isopropanol and placed the tube at -20°C for 10 minutes. Then, the DNA was centrifuged at 12,000 rpm for 10 minutes at 4°C and discarded the supernatant. The DNA pellet was washed with 1 ml of cold 70% ethanol and dissolved in 30 µl of TE buffer (pH 8.0).

The primer pairs (as shown in Table 2.1) were used for PCR detection of the integrated chitinase and NPTII gene. Amplification was done in volumes of 50 µl consisting of 1.5 mM $MgCl_2$, 0.2 mM dNTP, 25 pmole of each primer, 5 µl (about 100 µg/ml) of genomic DNA, and 0.5 unit of Taq polymerase (Phytotech Inc, Canada). Amplification of chitinase gene and NPTII fragments were performed for 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes, for denaturing, annealing, and primer extension, respectively.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Grape tissue culture

The grape cultivar Shiraz was used for meristematic bulk (MB) tissue for propagation. The MB tissue consisted of three sub-culturing phases. In the first phase, the apical domes of the shoots were maintained in IM1 culture medium supplemented with 4.4 μM BAP for 30 days (Figure 3.1A). In the second phase, the shoots were transplanted to IM2 medium containing 8.8 μM BAP and cultured for 30 days (Figure 3.1B). In the third phase, the proliferated shoots were placed to MM medium (13.2 μM BAP) and cultured for 30 days (Figure 3.1C). After 90 days, the MB could grow on MM medium and subcultured monthly on MM medium. This MB tissue can be easily propagated: the tissue is fragmented. Then each fragment generates a new MB tissue after 4-6 weeks of culture. Also, they were cut into small slice tissue. They can be transplanted for shoot and root inducing medium (MS medium supplemented with 0.5 μM NAA and 0.9 μM kinetin). Grape plants were obtained after 3-4 weeks (Figure 3.2A) and transferred into pots for plantlet (Figure 3.2B). Therefore, multiple shoot was induced and regenerated to plantlets for propagation totally about 4 months.

For this experiment, the multiple shoots have been induced for 3 months. However, Salunkhe et al. (1999) reported that a considerable longer shoot proliferation time, about 5 months. The callus formation was induced during any of multiplication stage. Multiplication using anther resulted in the formation of callus clumps at the base of shoot, which may be responsive to the long plantlet regeneration/

multiplication time. However, the plantlet could be induced form callus by using anther. However, the plantlet could be induced from callus by using anther. Singh et al. (2004) reported that two-node micro-cutting technique was suitable for multiplication of plantlet. The results showed that the proliferated shoot culture was achieved in 12 weeks cycle, which was in huge demand by grape grower for planting material.

In the present study, the above experiment was common used in a vegetative plant propagation and genetic transformation. The MB can be used for genetic transformation by cutting the MB tissue into small slice (1 cm^2, 2 mm thickness), tissue layer (Mezztti et al., 2002). The method was produced in a relatively short period of time (90 days) and was valuable for both vegetative plant propagation and genetic transformation.

3.2 Evaluation of damage ability of crude extract from *L. leucocephala* to *P. viticola* sporangia

Crude extract from *L. leucocephala* was taken for chitinase activity assay. Chitinase activity was determined by colorimetric assay. The obtained density was observed at 550 nm. The specific activity of leucaena chitinase was shown at 0.024 unit/mg proteins.

Crude extract was taken for testing the damage ability of chitinase to *P. viticola* sporangia. Sporangia suspension derived from a clone of *P. viticola* after being cultured for 7 days (shown in Appendix) in growth chamber. The numbers of normal sporangia were counted everyday under microscope (shown in Appendix). An appearance of normal and damaged sporangia was shown in Figure 3.3. Percentage of damaged sporangia was calculated as shown in Appendix and Figure 3.4.

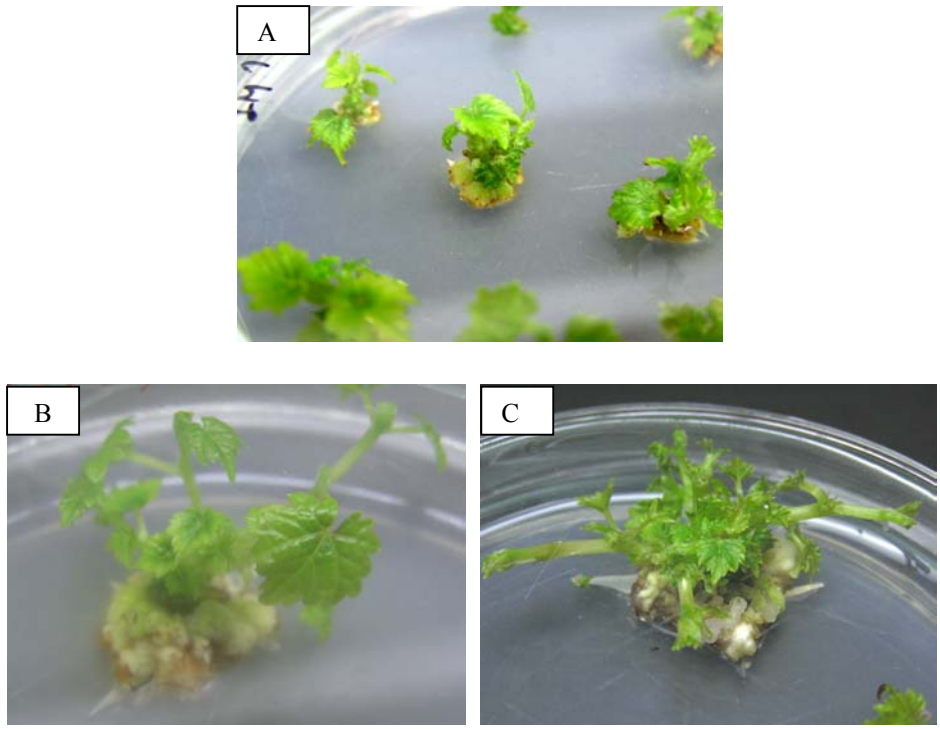


Figure 3.1 Meristematic bulk tissue (MB) regenerated from grape shoot; (A) The shoots were appeared after 30 days of culture; (B) Multiple shoots were appeared after 60 days; (C) Meristematic bulk tissue was generated after 90 days.

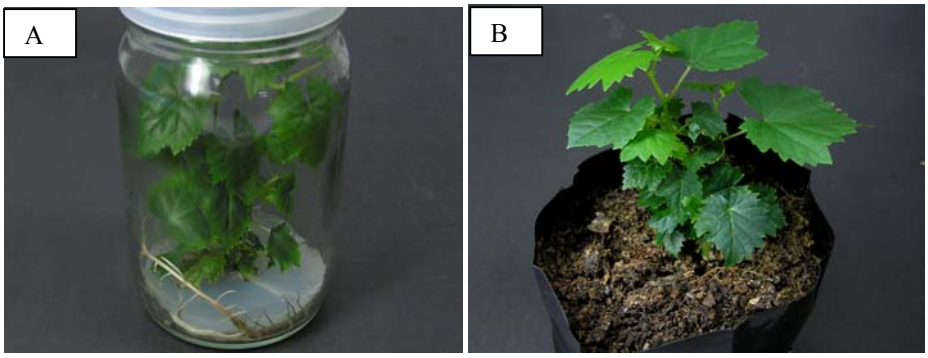


Figure 3.2 Shoot and root induced on MS medium (A) and grape plants were grown on pot (B).

According to the result, the percentages of damaged sporangia on day 3 were about 4%, 25%, and 55% of 0.011, 0.021, and 0.042 unit, respectively. Control and 0.01 unit of crude extract treatment were not significantly different, but 0.021 unit and 0.042 unit were significantly different on day 3 (shown in Appendix). Percentage of damaged sporangia was increased when the amount of enzyme increased. These results agreed with Mana Kaomek et al. (2003), that the recombinant chitinase from *L. leucocephala* exhibited antifungal activity by causing *in vitro* growth inhibition of 13 strains fungi. Also, Poonsuk Sriyotha and Peberdy (1996) reported that the crude chitinase enzyme from seeds of *Acacia mangium* and *Gliricidia sepium* could inhibit the growth of all the tested fungi. Those plant, belong to Leguminosae family as same as *L. leucocephala*. Therefore, these result demonstrated that the chitinase enzyme could damage of *P. viticola* sporangia, likely those which have chitin in cell wall.

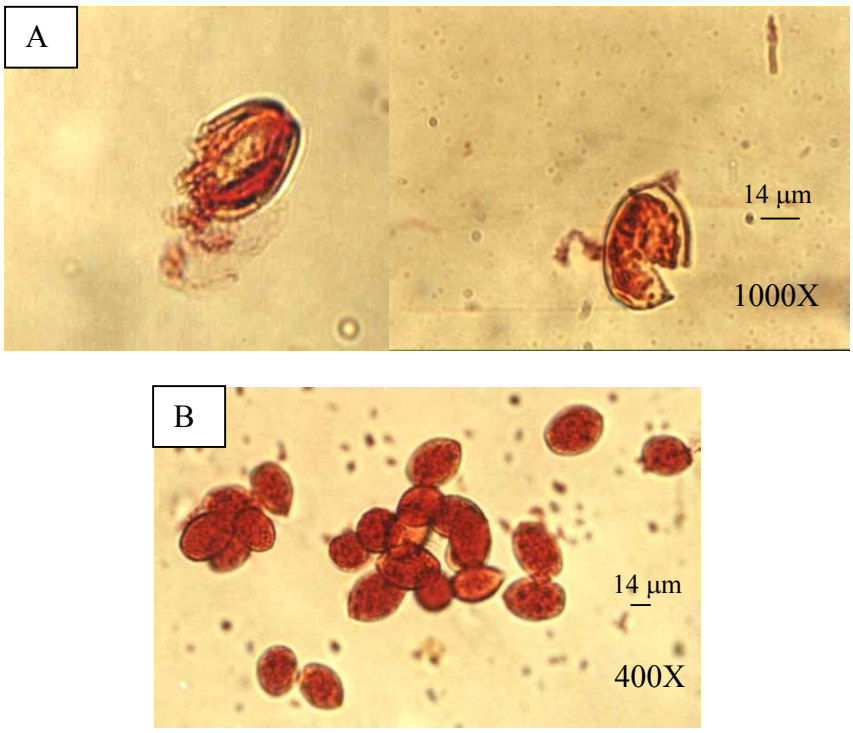


Figure 3.3 The appearance of damaged (A) and normal (B) *P. viticola* sporangia.

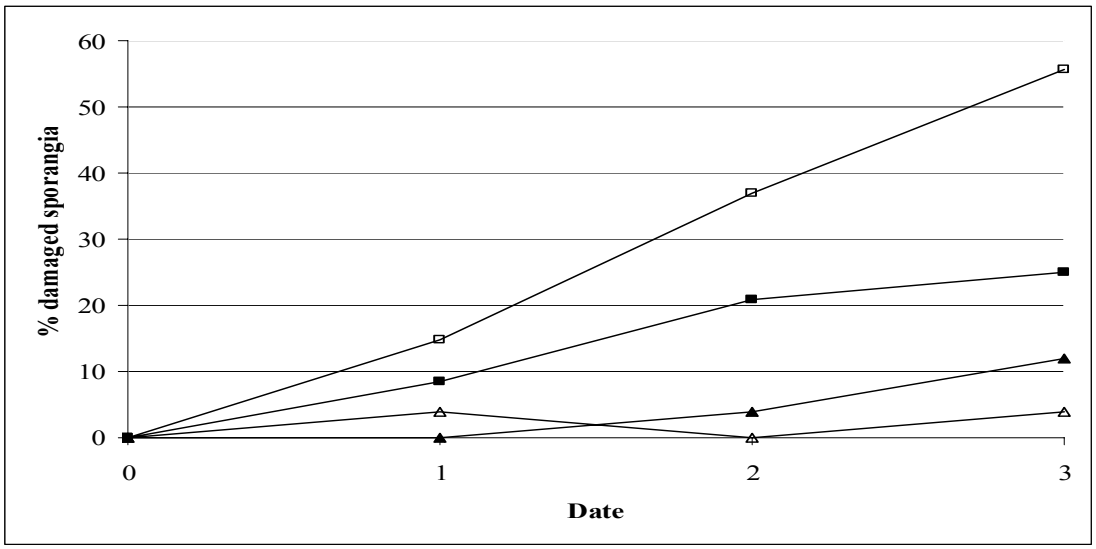


Figure 3.4 Percentage of damaged sporangia of *P. viticola* treated with crude extract of *L. leucocephala*; control (▲), 0.011 unit (△), 0.021 unit (■), and 0.042 unit (□).

3.2.1 Chitinase activity of grape

Crude extract from shoots, stem-internodes, and leaves of grapes were taken for chitinase activity assay. Chitinase activity was determined by colorimetric assay the obtained density was observed at 550 nm. The results were shown in Table 3.1. Root stock cultivar 1613 showed the highest activity related to the appearance of physiology, which could resist downy mildew. Crimson Seedless showed the lowest activity at 0.011 unit. Shiraz was low chitinase activity which was 0.014 unit related to the appearance of physiology. Since, grapevine cultivar Shiraz was susceptible for downy mildew. Thus, the Shiraz was chosen for genetic transformation for disease resistance and for modeling of other grape cultivars. As can be seen, the Shiraz is one of the most wildly planted red grape varieties in Thailand. It is very popular in wine making. Consequently, genetic transformation is one way to maintain the quality of Shiraz for red wine making.

Table 3.1 Specific activity of chitinase from various tissues of grapes.

	Specific activity (Unit/mg)		
Sample	**Leaves**	**Shoots**	**Stem internodes**
Loose Perlet	0.014	0.051	0.017
Crimson Seedless	0.001	0.053	0.076
Carolina Black Rose	0.056	0.025	0.083
Ruby red	0.075	0.128	0.221
Shiraz	0.014	0.085	0.025
Reisling	0.009	0.092	0.012
Root stock cultivar 1613	0.200	0.147	0.042

3.3 Cloning of chitinase gene into pET-39b(+), pBI121 vector and grape callus

3.3.1 Cloning of chitinase gene into pET-39b(+) vector

A chitinase encoding gene from pUC19 plasmid was used for chitinase in pET-39b(+) obtaining. The cloned amplified fragment plasmid insert was confirmed by restriction digest with *Eco*RI and *Nde*I. The chitinase size was analyzed on agarose gel electroporesis.

The recombinant plasmid contained 1.0 kb chitinase gene and 6.1 kb size of pET-39b(+) vector. The insert size was about 1.0 kb (lane 3) and 1.8 kb (lane 2) after being digested with *Eco*RI and *Nde*I, respectively (Figure 3.5). The cloned amplified fragment plasmid insert was digested by *Nde*I restriction to ensure the right direction of inserted chitinase gene (shown in Appendix). This indicated that chitinase encoding gene was transformed into pET-39b(+).

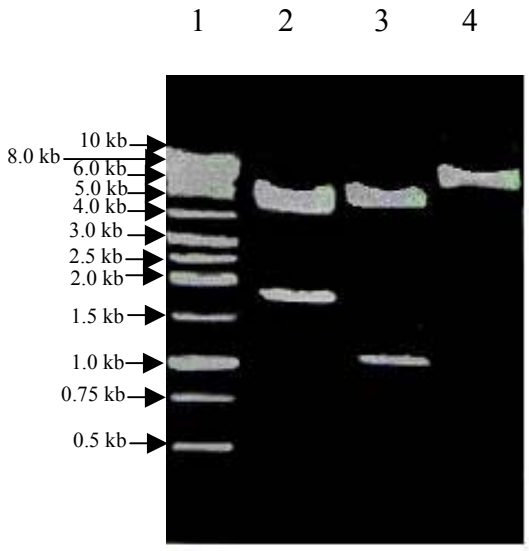


Figure 3.5 1% agarose gel electrophoresis of *Eco*RI and *Nde*I restriction enzyme digest of recombinant pET-39b(+) containing chitinase cDNA.

Lane 1: 1 kb DNA marker

Lane 2: *Nde*I digested recombinant plasmid

Lane 3: *Eco*RI digested recombinant plasmid

Lane 4: Undigested recombinant plasmid

3.3.2 Cloning of chitinase gene into pBI121 vector

The chitinase gene in pET-39b(+) vector was transferred to pBI121 expression vector. The single colony was picked from plates and DNA mini-prepared from pBI121 vector that integrated with chitinase gene. A pBI121 recombinant plasmid was digested by *Bam*HI and *Sac*I restriction enzyme.

The size of the recombinant plasmid was about 11.1 kb (lane 2) which shown the insert size about 1.1 kb (lane 3-5) of chitinase gene (Figure 3.6). According, the result illustrated that cDNA fragment of leucaena chitinase encoding gene was integrated into pBI121 vector and substituted the GUS gene (shown in Appendix).

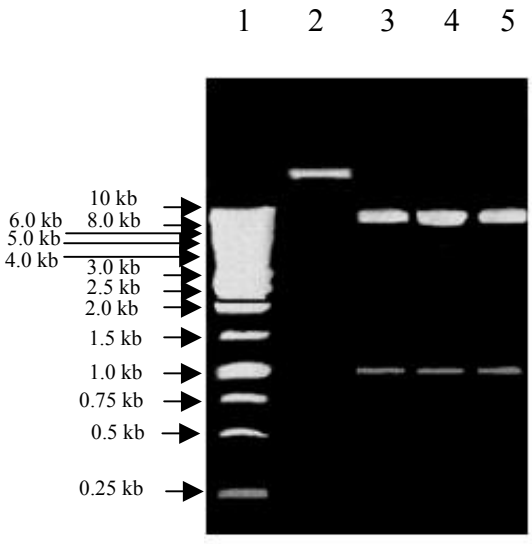


Figure 3.6 1% agarose gel electrophoresis of *Bam*HI and *Sac*I restriction enzyme digest of recombinant pBI121 containing chitinase cDNA.

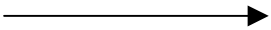
Lane 1: 1 kb DNA marker

Lane 2: Undigested recombinant plasmid

Lane 3-5: *Bam*HI and *Sac*I digested recombinant plasmid from each colony

3.3.3 Sequencing of pBI121: chitinase using chitinase forward primer

Oligonucleotide primers of chitinase forward were designed from terminal sequence of CaMV35s. The expected sequence of the pBI121: chitinase was confirmed by sequencing and analysis. The start codon of leucaena chitinase gene was subcloned into pBI121 at the *Bam*HI site. This result was shown in Figure 3.7. This indicates that leucaena chitinase encding gene was integrated into pBI121 with right direction.



CaMV35s Promoter……AGACCCTTCCTCTATCTAAAGGAAGTTCATTTCATTTGGA

Luecaena chitinase

*Xba*I *Bam*HI *Eco*RI 1

GAGAACACGGGGGACTCTAGAGGATCCGAATTCAAATCCAAGAGATGGATAAC 9
M D N 3

ATGAACAAGATGCGGGTGCTCTTGTGTGTGCTGCTGTACAGCTTCATGGTGGGA 63
M N K M R V L L C V L L Y S F M V G 21

GGCTTAGCGGAGCAATGCGGCAGGCAGGCAGGGGGTGCCTTGTGCCCCGGCCGC 117
G L A E Q C G R Q A G G A L C P G R 39

CTCTGCTGCAGCCAATTCGGCTGGTGTGGTTCCACTAACGATTACTGCGGCCCC 171
L C C S Q F G W C G S T N D Y C G P 57

GGTTGCCAGAGCCAGTGTGGCGGAAGTGGCCCAGGCCCAGCCCCTCCCTCCGGT 225
G C Q S Q C G G S G P G P A P P S G 75

GGCCTCACCGGCATCATCTCCAGGGACACCTTCAACCAGATGCTCAAGCACCGC 279
G L T G I I S R D T F N Q M L K H R 93

AACGACGCCGCCTGCCCGGCCAATGGCTTCTACACCTACGACGCCTTCATTCAG 333
N D A A C P A N G F Y T Y D A F I 111

GCCGCCAAGTCTTTCCCCGCCTTCGGTAGTACCGGCGATGATGCCACGCGCAAG 387
A A K S F P A F G S T G D D A T R K

AGGGAGGTGGCAGCTTTCCTCGGCCAAACTTCACACGAGACCACCGGCGGTTGG 441
R E V A A F L G Q T S H E T T G G W

CCCAGCGCTCCCCACGTCCTTACGCCTGGGGTTACTGCTTTCAAACAGGA……. 489
P S A P H V L T P G V T A F K Q E

……..NOS Terminator

Figure 3.7 Nucleotide and amino acid sequence of leucaena chitinase gene in pBI121.

3.3.4 Introduction of pBI121: chitinase plasmid into *Agrobacterium tumefaciences* LBA4404

A 3.0 kb of *Sac*I/*Bam*HI fragment containing GUS gene was deleted from the binary vector pBI121, the remaining other 10.0 kb large fragment containing T-DNA of the vector. The T-DNA contains NPTII coding region, conferring

kanamycin resistance as selectable marker.

The pBI121: chitinase gene was introduced into *A. tumefaciences* LBA4404 by the electroporation method. Electroporation was performed at 25 μF, the volts at 2.5 kV of Gene Pulser (Bio-rad). The clones of bacterial cells were picked and DNA mini-prepared for screening the chitinase gene in pBI121 vector. The chitinase gene was digested by *Bam*HI and *Sac*I to confirmed insert and plasmid DNA in *Agrobaterium*. The result from gel electrophoresis was about 1.1 kb of insert size and 10.0 kb of pBI121 vector (Figure 3.8). These results indicated that this cDNA fragment was from a leucaena chitinase.

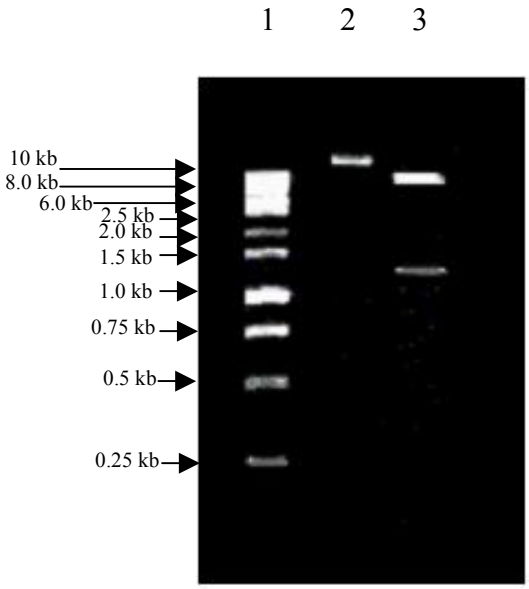


Figure 3.8 1% agarose gel electrophoresis of *Bam*HI and *Sac*I restriction enzyme digest of recombinant pBI121 containing chitinase cDNA.

Lane 1: 1 kb DNA marker

Lane 2: Undigested recombinant plasmid

Lane 3: *Bam*HI and *Sac*I digested recombinant plasmid

Electroporation was used for this experiment because it was found to be superior to the tri-parental mating procedure in the time required to introduce foreign DNA into *Agrobacterium* and also eliminated the contamination by *E. coli* strains (Singh et al., 1993). Moreover, the recombinant plasmid was directly transformed into the *A. tumefaciences* cells and less steps of manipulation. However, this experiment, the transformation efficiency was not high ($1.056x10^3$cfu/microgram). This is related to Lin, 2004 reported that the highest transformation efficiency (>$1.0x10^7$ cfu/μg) obtained from YM broth and YM plate. The increasing of the transformation efficiency of 40-fold was achieved by using both YM broth and YM plates compared to the efficiency using of LB broth and LB plates.

3.4 DNA extraction and detection of foreign gene in callus by PCR techniques

The grape leaf was co-cultivated with *A. tumefaciences* carring pBI121: chitinase vector and cultured on selective medium (NN medium supplemented with 250 mg/l cefotaxime, 250 mg/l carbenicillin and 100 mg/l kanamycin). Callus of the grape cultivar Shiraz were induced after 14 days (Figure 3.9A). The callus were grown for 1 month (Figure 3.9B) and 2 months (Figure 3.9C). They were taken for DNA extraction, and detected chitinase gene and NPTII gene by PCR analysis.

Genomic DNA was extracted by DNA mini-preparation method. Then, DNA was taken for PCR analysis. Four clones were analyzed to confirm the existence of chitinase gene and NPTII gene (Figure 3.10). Transgenic grape callus were produced by using an *Agrobaterium*-mediated transformation. After 14 days, the callus were induced on selective medium supplemented with 250 mg/l cefotaxime, 250

mg/l carbenicillin, and 100 mg/l kanamycin and extracted for PCR techniques. The chitinase and NPTII gene were recovered in all of 4 clones (about 1.1 kb and 0.8 kp size, respectively). The results indicated that this cDNA fragment was transformed into grape callus, related to Yamamoto et al. (2000). The rice chitinase gene was introduced into the grapevine plants. Ovules of grapevine cultivar Neo Muscat were used for callus induction and applied for *Agrobaterium*-mediated transformation. Germinated transgenic plantlet was obtained by culturing on the selective medium for 4 months after co-cultivation. Transformants were compared with those of the non-transformant under scanning electron microscope. The conidia germination mycerial growth, and conidial formation of *U. necator* were suppressed on the leaf surface of the transformant. Mezzetti et al. (2002) reported that transgenic grape plant (*V. vinifera*, table grape cultivars Silcora and Thomson Seedless) was used an *Agrobacterium*-mediated transformation. About 7 months following the initiation of Meristematic Bulk tissue culture, transgenic grape plant of these two grape cultivars were obtained, which related with Nakano et al. (1994). Callus were induced from leaf of grapevine (*V. vinifera* L. cultivar Koshusanjaku) and co-cultivated with *A. rhizogenes*. After one month, callus were taken for confirming the transformation. The callus showed that all of them containing opine and somatic embryo-derived plantlets were successively sub-cultured by terminal cutting to cefotaxime-containing NHF-medium in order to eliminate *Agrobacterium.*

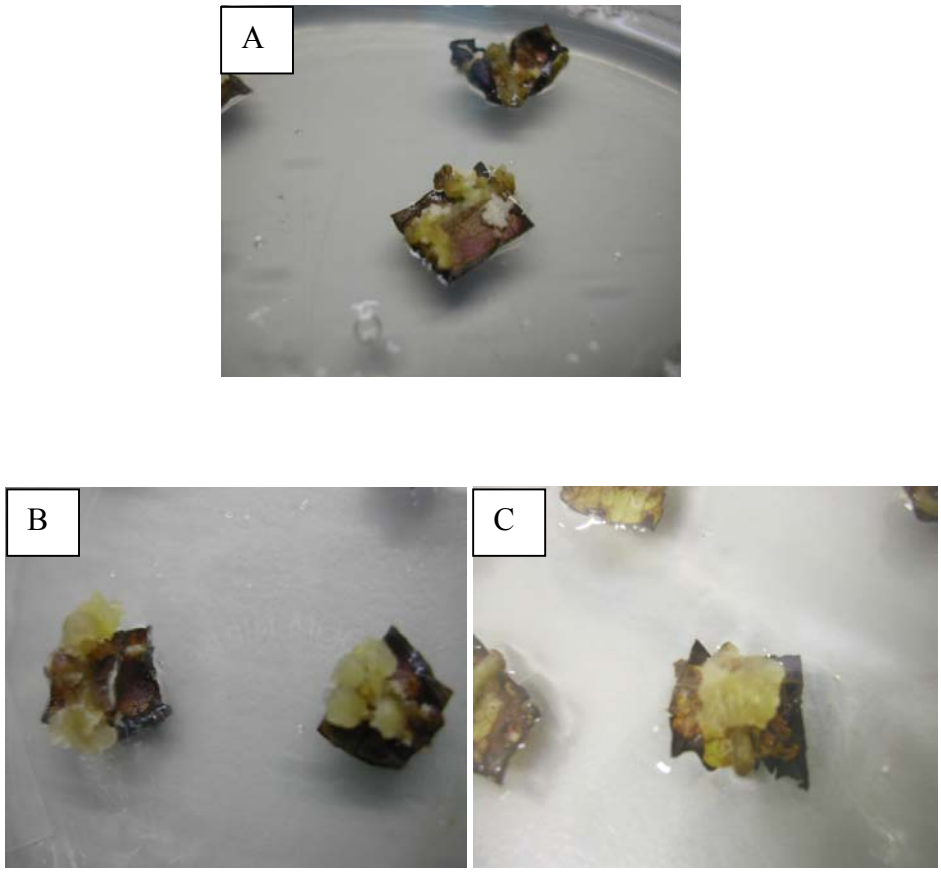


Figure 3.9 The grape callus cultivar Shiraz on selective medium (A) for 14 days, (B) 1 month, and (C) 2 months.

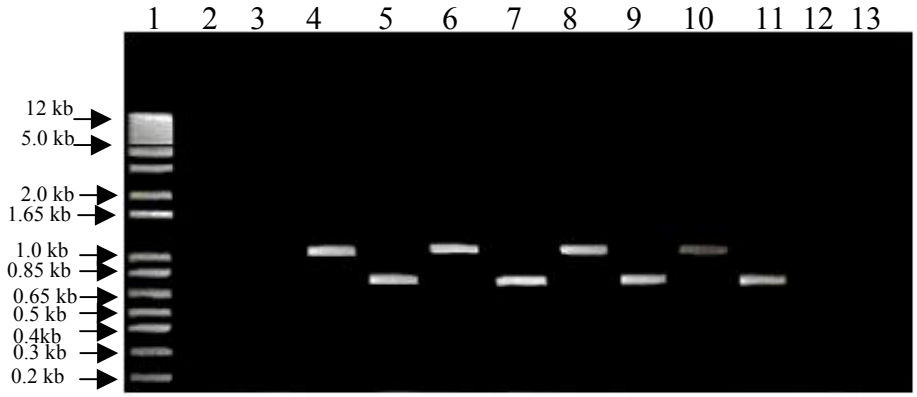


Figure 3.10 PCR products from chitinase and NPTII primers of transformants on 1% agarose gel electrophoresis.

Lane 1: 1 kb DNA Ladder marker

Lane 2: Grapevine untransformant with chitinase primer

Lane 3: Grapevine untransformant with NPTII primer

Lane 4, 6, 8, and lane10: Grapevine transformant with chitinase primer

Lane 5, 7, 9, and lane11: Grapevine transformant with NPTII primer

Lane 12: Negative control with chitinase primer

Lane 13: Negative control of NPTII primer

CHAPTER IV

CONCLUSION

Grapes (*Vitis* species) are cultivated commercially in more than 60 countries, including Thailand. Downy mildew, *P. viticola*, is a major fungal disease of grapevine that causes extensive losses in yield and quality. Multiple shoot induction was successful by using IM and MM media supplemented with NAA and BAP. Moreover, the sporangia of *P. viticola* were damaged by crude extract from *L. leucocephala.* Transgenic grape plant is the way to improve resistance ability of grapevine to downy mildew. In experiment, the transgenic grape callus were obtained by using *Agrobacterium*-mediated transformation. The chitinase gene from *L. leucocephala* was successfully transformed to *E.coli* by using pET-39b(+) and pBI121 as expression vector. The electroporation technique was useful for chitinase gene transformation to *Agrobacterium*. The *Agrobacterium* carrying chitinase gene infected into plant, and transferred chitinase gene to plant tissue and induced to be callus. The chitinase gene was successfully transformed to grape callus and proved by PCR method. The chitinase gene and NPTII gene (about 1.1 kb and 0.8 kp) were found in the four clones of grape callus. The results indicated that *Agrobacterium*-mediated transformation could be used for gene transformation in grapevine. Moreover, the NN medium supplemented with 2,4-D and 4-CPPU were appropriate for callus induction within 14 days.

REFERENCES

Bordoli, L., Falquet, L., and Ioannisdis, B. (2004). **iPCR product extractor** [On-line]. Available: http://www.ch.embnet.org/software/iPCR_form.html

Balasubramaniam, R., Harvey, I.C., Braithwaite, M., and Jordan, D. (2005). **Grapevine disease in New Zealand** [On-line]. Available:http://www.hortnet.co.nz/publications/hortfacts/images/90502003.gif

Bartnicki-Garoia, S. (1968). Cell wall chemistry, morphologenesis, and taxonomy of fungi. **Annu. Rev. Microbial**. 22:87-108 Quoted in Kaomek M. (2001). **Cloning, expression, and usage of chitinase form *Leucaena leucocephala* de Wit**. Ph.D. Dissertation, Suranaree University of Technology, Thailand.

Bobadoost, M. (2001). **Report on plant disease RPD No. 705: Downy mildew of grape.** USA: The University of Illinois.

Boonkerd N. 2000. **Manual of vineyard.** Nakhon Ratchasima: Suraree University of Technology.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., and Vad, K. (1993). Plant chitinase. **The Plant J.** 3(1): 31-40.

Datta, S.K., and Muthukrishnan, S. (1999). **Pathogenesis-related proteins in plants.** USA: CRC press.

Ellis, M.A. (2004). **Plant pathology** [On-line]. Available: http://www.ohioline.ag.ohiostate.edu.html

Ellis, M.A., and Nita, M. (2005). **Integrated management of grape diseases** [On-line]. Available: http://www.oardc.ohio-state.edu/fruitpathogy/organic/grape/All-Grapes.html

Emershad, R.L., and Ramming, D.W. (1994). Somatic embryogenesis and plant development from immature zygotic embryos of seedless grapes (*Vitis vinifera* L.). **Plant Cell Rep.** 14: 6-12.

Harst, M. (1995). Development of a regeneration protocol for high frequency somatic embryogenesis from explants of grapevine (*Vitis* spp.). **Vitis.** 34(1): 27-29.

Harst, M., Bornhoff, B.A., Zyprian, E., and Topper, R. (2000). Influence of culture technique and genotype on the efficiency of *Agrobacterium*-mediated transformation of somatic embryos (*Vitis vinifera*) and their conversion to transgenic plants. **Vitis.** 36(3): 99-102.

Hahn, M., Hennig, M., Schlesier, B., and Hohne, W. (2000). Structure of jack bean chitinase. **Acta Cryst.** 56: 1096-1099.

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. **Biochem. J.** 280:309. Quoted in Datta S.K., and Muthukrishnan, S. (1999). **Pathogenesis-related proteins in plants.** New York: CRC press.

Hanahan, D. (1968). Studies on transformation of *Escherichia coli* with plasmid. **J.Mol.Biol**. 166: 557-560 Quoted in Sambrook, J. And Russell D.W. (2001). **Molecular cloning: a laboratory manual**. New York: CSHL Press.

Huynh, Q.K., Hironaka, C.M., Levine, E.B., Smith, C.E., Borgmeyer, J.R., and Shah, D.M. (1992). Antifungal proteins from plants. **The J. Bio. Chem.** 267: 6635-6640.

Ivie International. (2003). **World vineyard, grape and wine report 2003: World wine production 1951-2001.** California: Ivie International.

Jayasankar, S., Gray, D.J., and Litz, R.E. (1999). High-efficiency somatic embryogenesis and plant regeneration from suspension cultures of grapevine. **Plant Cell Rep.** 18: 533-537.

Jollès, P., and Muzzarelli, R.A.A. (1999). **Chitin and Chitinases.** Berlin: Birkhauser Verlag Press.

Kaomek, M., Mizuo, K., Fujimura, T., Sriyotha, P., and Ketudat Cairns, J. (2003). Cloning, Expression, and Characterization of an Antifungal Chitinase from *Leucaena leucocephala* de Wit. **Biosci. Biotechnol. Biochem.** 67(2):667-676.

Kikkert, J.R., Ali, G.S., Striem, M.J., Martens, M.H., Wallace, P.G., Molino, L., and Reisch, B.I. (1997). Genetic engineering of grapevine (*Vitis* sp.) for enhancement of disease resistance. **Acta Hort.** 447: 273-279.

Kikkert, J.R., Ali, G.S., Wallance, P.G., and Reisch, B. (2000). Expression of a fungal chitinase in *V. vinifera* L. "Merlot" and "Chadonay" plants produced by biolistic transformation. **Acta Hort.** 528: 297-303.

Kikkert, J.R., Hebert-soule, D., Wallance, P.G., Striem, M.J., and Reisch, B.I. (1996). Transgenic plantlets of Chancellor grapevine (*Vitis* sp.) from biolistic transformation of embryogenic cell suspensions. **Plant Cell Rep.** 15: 311-316.

Krishnaveni, S., Liang, G.H., Muthukrishnan, S., and Manickam, A. (1999). Purification and partial characterization of chitinase from sorghum seeds. **Plant Sci.** 144: 1-7.

Leo, S.M., et al. (1994). A new class of tobacco chitinase homologous to bacterial exo-chitinase displays antifungal activity. **The Plant J.** 5(4): 469-480.

Lin, J-J. (2004). A new expression medium for *Agrobacterium tumefaciens* following electroporation. **Focus.** 16(1): 18-19.

Lin, R.C., Ding, Z.S., Li, L.B., and Kuang, T.Y. (2001). A rapid and efficiency DNA minipreparation suitable for screening transgenic plants. **Plant Mol. Bio. Reporter**. 19: 379a-379e.

Marchenko, A.O. (1991). Induction of embryogenesis in primary calluses from grape stem and leaves. **Fiziologiya Rastenii.** 38(3): 580-590.

Mhatre, M., and Salunkhe, C.K. (2000). Micropropagation of *Vitis vinifera* L: towards an improved protocol. **Scientia Horticultureae.** 84: 357-363.

Mezzetti, B., Pandolfini,T., Navacchi, O., and Landi, L. (2002). Genetic transformation of *Vitis vinifera* via organogenesis. **BMC Biotech.** 2(18): 1-10.

Nakano, M., Hoshino, Y., and Mii, M. (1994). Regeneration of transgenic plants of grapevine (*Vitis vinifera* L.) via *Agrobacterium rhizogenes*-mediated transformation of embryogenic calli. **J. Experimental Botany.** 45(274): 649-656.

Papademetriou, M.M., and Dent, F.J. (2001). **Grape production in the Asia-Pacific region.** Bangkok: RAP Publication.

Pearson, R.C., and Goheen, A.C. (1988). **Compendium of grape disease.** USA: APS press.

Ponstein, A.S., Sela-Buurlage, M.B., Bres-Vloemans, S.A., Melchers, L.S., Van Der Elzen, P.J.M., and Corelissen, B.J.C. (1993). Only specific Tobacco (*Nicotiana tabacum*) chitinase and β–1,3-glucanases exhibit antifungal activity. **Plant Physiol.** 101: 857-863.

Rohini, V. K. and Rao, K.S. (2001). Transformation of peanut (*Arachis hypogaea* L.) with tobacco chitinase gene: variable response of transformants to leaf spot disease. **Plant Sci.** 160: 889-898.

Salunkhe, C. K., Rao, P. S., and Mhatre, M. (1999). Plantlet regeneration via somatic embryogenesis in anther callus of *Vitis latifola* L. **Plant Cell Reports**. 18: 670-673.

Salunkhe, C. K., Rao, P. S., and Mhatre, M. (1997). Induction of somatic embryogenesis and plantlet in tendril of *Vitis vinifera* L. **Plant Cell Rep.** 17: 65-67.

Sambrook, J., and Russell, D.W. (2001). **Molecular cloning: A laboratory manual.** USA: CSHL press.

Scorza, R., Cordts, J.M., Ramming, D.W., and Emershad, R.L. (1995). Transformation of grape (*Vitis vinifera* L.) zygotic-derived somatic embryos and regeneration of transgenic plants. **Plant Cell Rep.** 14: 589-592.

Sigh, A., Kao, T.H., and Lin, J.J. (1993). **Focus** 15, 84. Quoted in Lin J.J (2004). A new expression medium for *Agrobacterium tumefaciens* following electroporation. **Focus.** 16(1): 18-19.

Singh, S. K., Khawale, R. N., and Sigh, S. P. (2004). Technique for rapid in vitro multiplication of *Vitis vinifera* L. cultivars. **J. Hort. Sci. & Biotech.** 79(2): 267-272.

Sriyotha P., and Peberdy, J. 1996. **Research report: Chitin degrading enzymes and their catalytic proproties.** Nakhon Ratchasima: Suranaree University of Technology.

Tebei, Y., et al. (1998). Transgenic cucumber plants harboring a rice chitinase gene exhibit enhanced resistance to gray mold (*Botrytis cinerea*). **Plant Cell Rep.** 17: 159-164.

Verburg, J.G., and Huynh, Q.K. (1991). Purification and characterization of an antifungal chitinase from *Arabidopsis thaliana*. **Plant Physiol.** 95: 450-455.

Vilaplana, M., and Mullins, M. G. (1989). Regeneration of grapevines (*Vitis* spp.) in vitro: Formation of adventitious buds on hypocotyls and cotyledons of somatic embryos. **J. Plant Physiol.** 134: 413-419.

Wessels, J.H.G., and Sietma, J.H. (1981). Fungi cell well: A survey. In Tanner, W., and Loewus, F.A., (eds.). **Encyclopedia of plant physiology (volume 13B),** pp. 352-394. Berlin: Springer-Verlag.

Weaver, R.J. (1976). **Grape growing.** New York: John Wiley& Sons.

Yamamoto, T., et al. (2000). Transgenic grapevine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. **Plant Cell Rep.** 19: 639-646.

Yang, Z.N., Ingelbrecht, I.L., Louzada, E., Skaria, M., and Mirkov, T.E. (2000). *Agrobacterium*-mediated transformation of the commercially important grapefruit cultivar Rio Red (*Citrus paradisi* Macf.). **Plant Cell Rep.** 19: 1203-1211.

APPENDIX

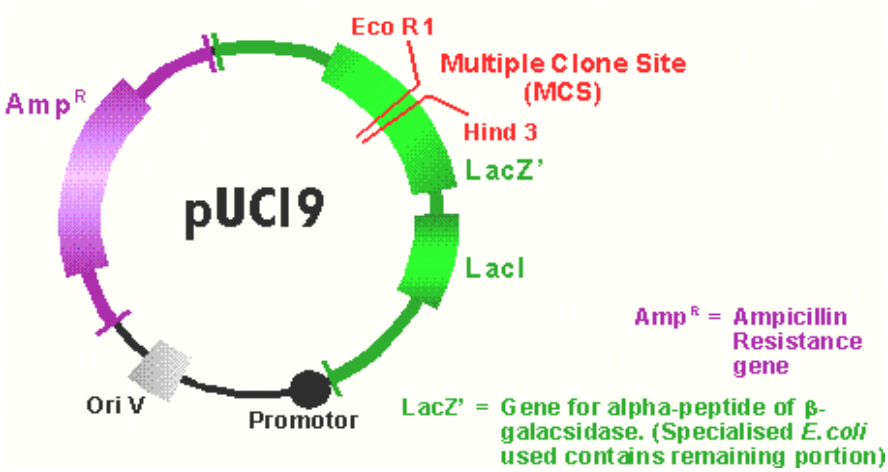


Figure A1 Diagram of pUC19 vector.

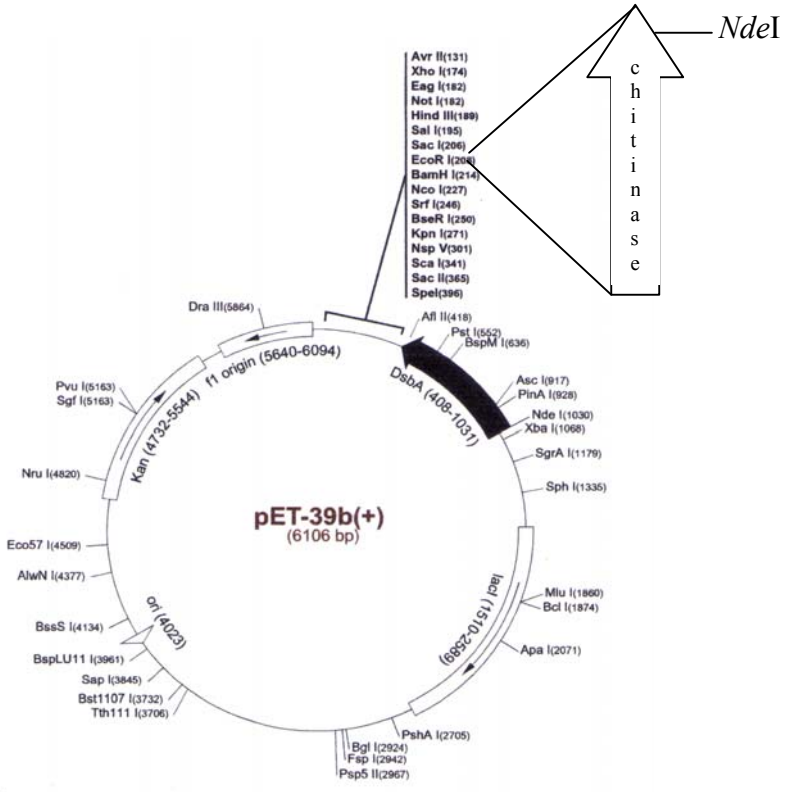


Figure A2 Diagram of pET-39b(+) vector with chitinase gene.

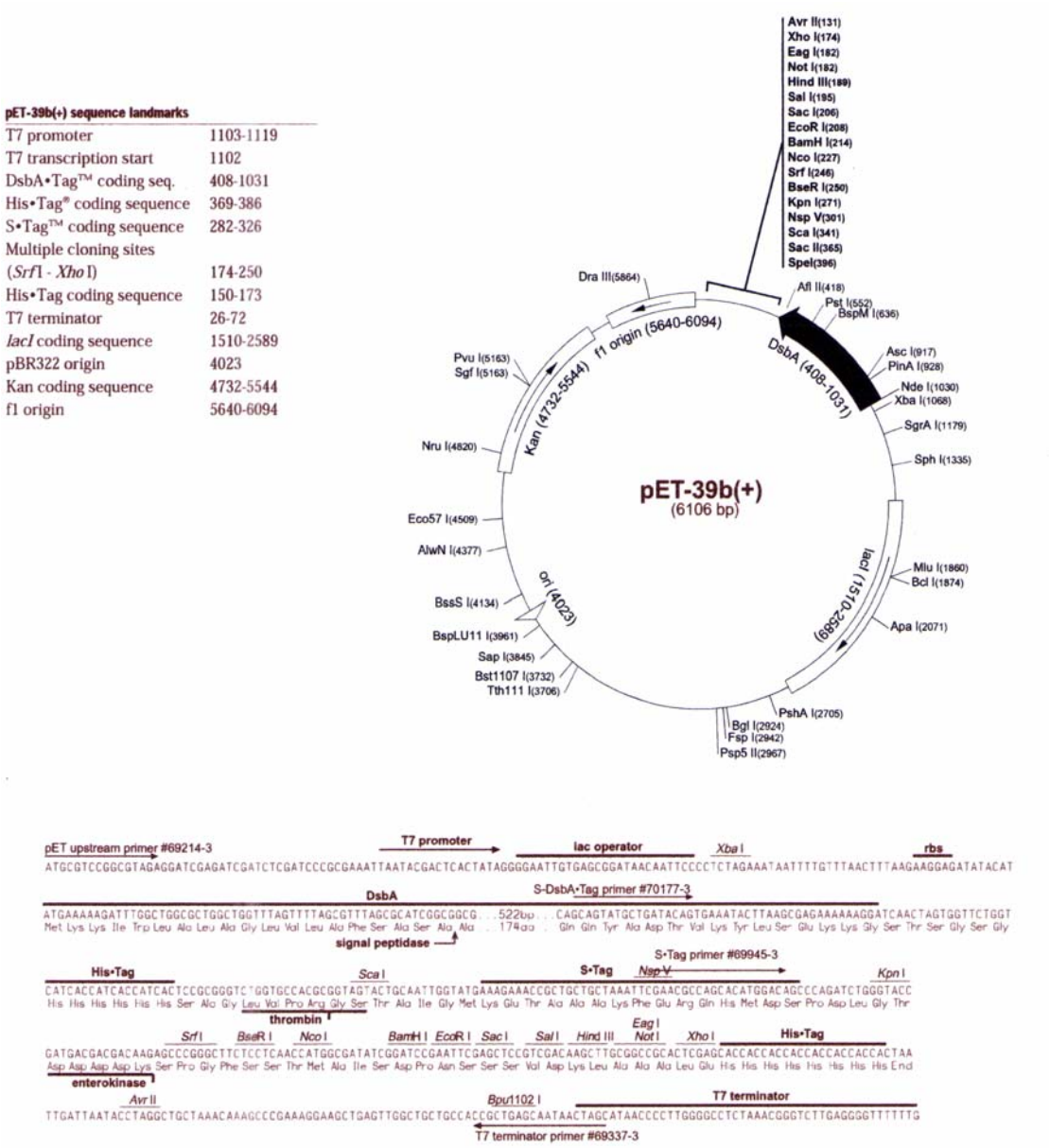


Figure A3 Diagram of pET-39b(+) vector.

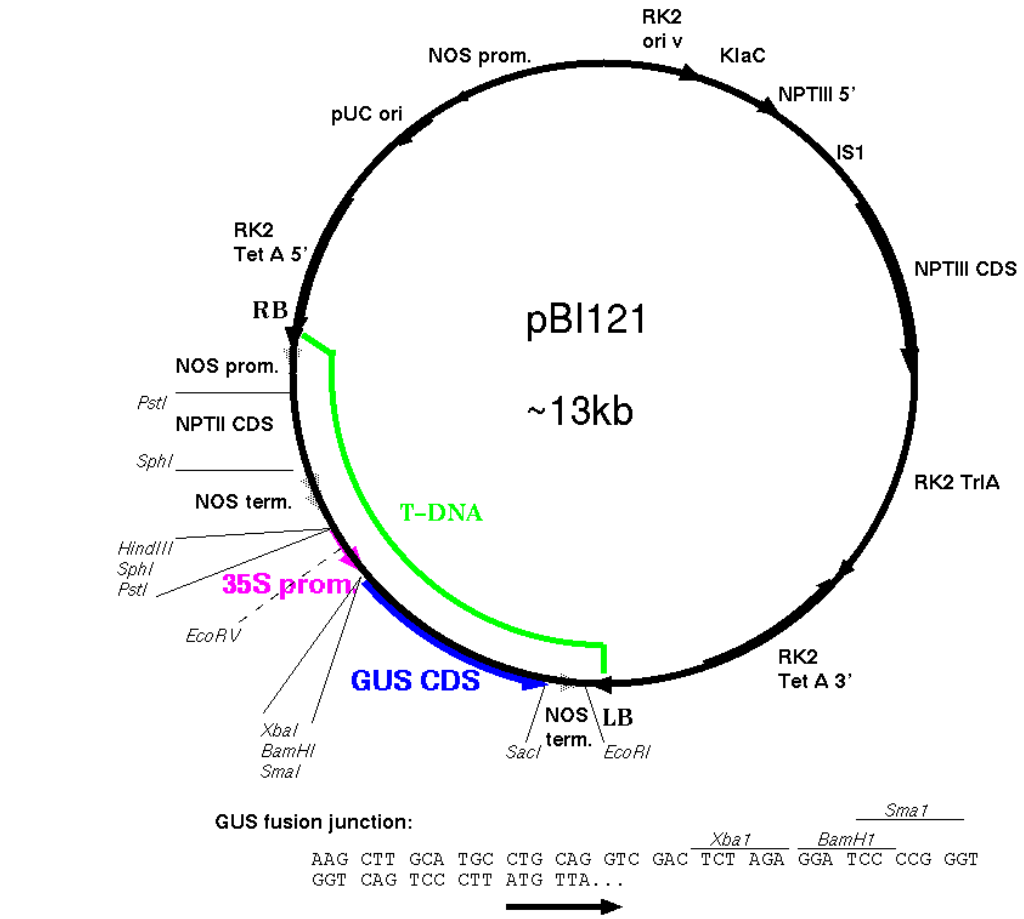


Figure A4 Diagram of pBI121 expression vector.

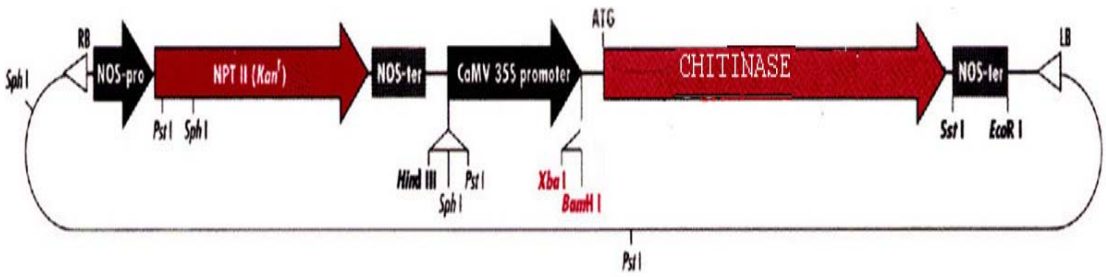


Figure A5 Diagram of the constructed plasmid pBI121-chitinase.

Figure A6 *P. viticola* cultured on grape leaves in box.

Figure A7 *P. viticola* cultured on grape leaves for 7 days.

Table A1 The number of normal and percentage of damaged sporangia of *P. viticola* treated with crude extract.

Date	O		1		2		3	
Sample (Unit)	Normal sporangia /ml(x10^4)	% damaged sporangia	Normal sporangia /ml (x10^4)	% damaged sporangia	Normal sporangia /ml (x10^4)	% damaged sporangia	Normal sporangia /ml (x10^4)	% damaged sporangia
Control[(1)]	1.67	0.00	1.73	0.00[a]	1.60	4.00[a]	1.47	12.00[a]
0.011	1.76	0.00	1.60	8.86[a]	1.67	5.06[a]	1.60	8.86[a]
0.021	1.60	0.00	1.47	8.33[a]	1.27	20.83[a]	1.20	25.00[ab]
0.042	1.80	0.00	1.53	14.81[a]	1.13	37.04[a]	0.80	55.56[b]

[(1)] Means in a columns followed by the different letter are significantly different (P ≥0.05)

BIOGRAPHY

Krongjai Tasing was born in Maha Sarakham, Thailand on September 23, 1975. She studied in primary school and high school at Khumuangwittayacom School. In 1994, she studied in School of Crop Production Technology, Suranaree University of Technology (SUT), Nakhon Ratchasima. She participated in the Co-operative Education Program to work as Quality Assurance Supervisor at Pioneer Hi-Bred (Thailand) Co. Ltd., Lampoon. She graduated the Bachelor's degree of science in Crop Production Technology in 1998. After graduation, she worked as research assistant for 1 year and 8 months in project "The Climatic variability and rice production in rainfed rice area in the northeast of Thailand" at SUT with Asst. Prof. Dr. Hutsachai Boonjung and worked as research assistant for 1 year in project "Grape Variety collecting and Testing, Nutrient Management and Vineyard Making" at SUT with Prof. Dr. Nantakorn Boonkerd. In 2001, she was Master's student in the field of Biotechnology at SUT. During Master's student, she had an experience on oral presentation in title "The transformation of chitinase into grape" at the 4th National symposium on Graduate Research, August 10-11, 2004, Lotus Pang Suan Kaew Hotel, Chiangmai, Thailand.