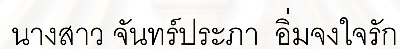
การทรานส์ฟอร์มรีพอร์เทอร์ยีนที่สร้างโปรตีนกรีนฟลูออเรสเซนต์เข้าสู่ข้าวพันธุ์ขาวดอกมะลิ 105
*Oryza sativa* cv. KDML 105 โดยเลี้ยงร่วมกับอะโกรแบคทีเรียม



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จุฬาลงกรณ์มหาวิทยาลัย

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TRANSFORMATION OF GREEN FLUORESCENT PROTEIN REPORTER GENE INTO RICE *Oryza sativa* cv. KDML 105 BY CO-CULTIVATION WITH AGROBACTERIUM

Miss Chanprapa Imjongjirak

สถาบันวิทยบริการ
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จันทร์ประภา อิ่มจงใจรัก : การทรานส์ฟอร์มรีพอร์เทอร์ยีนที่สร้างโปรตีนกรีนฟลูออเรสเซนต์เข้าสู่ข้าวพันธุ์ขาวดอกมะลิ 105 *Oryza sativa* cv. KDML 105 โดยเลี้ยงร่วมกับอะโกรแบคทีเรียม (TRANSFORMATION OF GREEN FLUORESCENT PROTEIN REPORTER GENE INTO RICE *Oryza sativa* cv. KDML 105 BY CO-CULTIVATION WITH AGROBACTERIUM) อ. ที่ปรึกษา: ผศ. นภา ศิวรังสรรค์ 117 หน้า. ISBN 974-346-541-3.


การทรานส์ฟอร์มรีพอร์เทอร์ยีนที่สร้างโปรตีนกรีนฟลูออเรสเซนต์ (GFP) เข้าสู่ข้าวพันธุ์ขาวดอกมะลิ 105 ทำได้โดยการเลี้ยงเอมบริโอเจนิคแคลลัส ที่ได้จากสคิวเทลลัมของเอมบริโอร่วมกับอะโกรแบคทีเรียม สายพันธุ์ *Agrobacterium tumefaciens* EHA105 ซึ่งมี pCAMBIA1301 (มียีนต้านยาไฮโกรไมซิน (*hpt*) เป็นยีนเครื่องหมาย และมีรีพอร์เทอร์ยีนคือยีนที่สร้าง GUS (*uidA* gene)) pCAMBIA5305 (มียีน *hpt* เป็นยีนเครื่องหมาย และมีรีพอร์เทอร์ยีนคือยีนที่สร้างโปรตีนกรีนฟลูออเรสเซนต์ (GFP)) และ pCAMBIA1306IC (มียีน *hpt* เป็นยีนเครื่องหมาย และมียีนที่สร้าง GUS และ GFP เป็นรีพอร์เทอร์ยีน) โดยยีนเหล่านี้ถูกควบคุมด้วยโปรโมเตอร์ CaMV35S

จากการทดลองชักนำเมล็ดข้าวขาวดอกมะลิ 105 ให้เกิดเอมบริโอเจนิคแคลลัสบนอาหาร 2 สูตร คือ 2MS และ 2NB พบว่าสูตร 2NB สามารถชักนำให้เกิดเอมบริโอเจนิคแคลลัสได้ 79.2±3.4% ในขณะที่ 2MS ชักนำให้เกิดได้ 54.6±2.5% เมื่อนำแคลลัสที่ได้มาทำให้เกิดเป็นต้นโดยเปรียบเทียบอาหาร 4 สูตร คือ MS1-RE NB1-RE NB2-RE และ NB4-RE พบว่าอาหารสูตร NB4-RE สามารถชักนำให้เกิดยอดได้ดีที่สุด คือ 68.2±13.6% ในการทรานส์ฟอร์มโดยใช้อะโกรแบคทีเรียม เริ่มแรกได้นำอะโกรแบคทีเรียมที่มี pCAMBIA1301 มาเลี้ยงร่วมกับเอมบริโอเจนิคแคลลัสเป็นเวลา 3 วัน และตรวจสอบการทรานส์ฟอร์มโดยการแสดงออกของรีพอร์เทอร์ยีนที่สร้าง GUS พบว่ามีเปอร์เซนต์การทรานส์ฟอร์ม 15.8% และหลังจากเลี้ยงร่วมกันเป็นเวลา 3 วันแล้ว ได้นำแคลลัสลงเลี้ยงในอาหารที่มีไฮโกรไมซิน (50 mg/l) พบว่ามีแคลลัสที่สามารถต้านทานไฮโกรไมซินเกิดขึ้นหลังจาก 4 สัปดาห์ แคลลัสที่ไม่ได้เลี้ยงร่วมกับอะโกรแบคทีเรียมไม่สามารถเจริญได้ในอาหารที่มีไฮโกรไมซิน หลังจาก 8 สัปดาห์พบว่าเปอร์เซนต์การทรานส์ฟอร์มของแคลลัสที่สามารถเจริญได้ในไฮโกรไมซิน และมีการแสดงออกของรีพอร์เทอร์ยีนที่สร้าง GUS คือ 19.4% ในการทรานส์ฟอร์มรีพอร์เทอร์ยีนที่สร้าง GFP เข้าสู่ข้าวสามารถทำได้โดยใช้อะโกรแบคทีเรียมที่มี pCAMBIA5305 และ pCAMBIA1306IC มาเลี้ยงร่วมกับเอมบริโอเจนิคแคลลัสเป็นเวลา 3 วันเช่นเดียวกัน แคลลัสที่ได้รับการทรานส์ฟอร์มรีพอร์เทอร์ยีนที่สร้าง GFP จะถูกชักนำให้เกิดเป็นต้นใหม่ การตรวจสอบการทรานส์ฟอร์มรีพอร์เทอร์ยีนที่สร้าง GFP เข้าสู่ข้าว ทำได้โดยตรวจการเรืองแสงของ GFP ในต้นข้าวและตรวจสอบยีนของ GFP ด้วยเทคนิคพีซีอาร์ พบว่าสามารถตรวจผลิตผลพีซีอาร์ได้ในทุกต้นของข้าวที่มีการเรืองแสงของ GFP และพบว่าประสิทธิภาพของการทรานส์ฟอร์มของอะโกรแบคทีเรียม เข้าสู่ต้นข้าวที่มีการแสดงออกของ GFP ที่ได้จากการทรานส์ฟอร์มด้วย pCAMBIA5305 และมีทั้งการแสดงออกของ GFP และการแสดงออกของรีพอร์เทอร์ยีนที่สร้าง GUS ที่ได้จากการทรานส์ฟอร์มด้วย pCAMBIA1306IC มีค่า 9.8% และ 8.2% ตามลำดับ



ภาควิชา……………ชีวเคมี…………………… ลายมือชื่อนิสิต ....................................................

สาขาวิชา…………..ชีวเคมี…………………… ลายมือชื่ออาจารย์ที่ปรึกษา ....................................

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*Agrobacterium*-mediated transformation was used to transfer green fluorescent protein (GFP) reporter gene into indica rice variety (*Oryza sativa* cv. KDML 105). Embryogenic calli derived from scutellum of mature embryo were co-cultivated with *Agrobacterium tumefaciens* strain EHA105 carrying pCAMBIA1301 (hygromycin resistance gene (*hpt*) as a selectable marker and GUS (*uidA* gene) as the reporter gene), pCAMBIA5305 (*hpt* as a selectable marker and GFP as the reporter gene) and pCAMBIA1306IC (*hpt* as a selectable marker and GFP and GUS as the reporter gene). Each was driven under the CaMV35S promoter.

Mature embryos were cultured on 2MS and 2NB medium for callus induction. 2NB medium gave 79.2±3.4% embryogenic calli induction, higher than 2MS medium (54.6±2.5%). The embryogenic callus was transferred to four regeneration medium (MS1-RE, NB1-RE, NB2-RE and NB4-RE. High percentage (68.2±13.6%) of plantlet regeneration was obtained from the NB4-RE medium. Embryogenic calli were co-cultivated with *Agrobacterium*. Primarily, GUS expression from pCAMBIA1301 in the callus stage was examined immediately after 3 days of co-cultivation revealed 15.8% transformation efficiency. Selection was made on selective medium containing 50 mg/l hygromycin. Hygromycin resistant calli were obtained after 4 weeks selection. The uninoculated calli did not show continuous growth and died. After 8 weeks, the frequency of transformed calli, based upon hygromycin resistance and GUS activity was 19.4%. Use of *A. tumefaciens* strain EHA105 (pCAMBIA5305) and EHA105 (pCAMBIA1306IC), GFP transformed calli were obtained and regenerated. The presence of GFP gene was confirmed by PCR analysis and detection of GFP fluorescence. The transformation frequency obtained in this work determined by plants that stably expressed GFP for pCAMBIA5305 and expressed both GFP fluorescence and GUS activity for pCAMBIA1306IC was 9.8% and 8.2%, respectively.

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# LIST OF ABBREVIATION

| | | |
|---|---|---|
| ATP | = | adenosine triphosphate |
| bp | = | base pair |
| $^{o}C$ | = | degree celcius |
| CTAB | = | cetyltrimethylammonium bromide |
| CAT | = | chloramphenicol |
| dATP | = | deoxyadenosine triphosphate |
| dCTP | = | deoxycytosine triphosphate |
| dGTP | = | deoxyguanosine triphosphate |
| dTTP | = | deoxythymine triphosphate |
| DNA | = | deoxyribonucleic acid |
| FITC | = | fluorescein isothiocyanate |
| GFP | = | green fluorescent protein |
| GUS | = | β-glucuronidase |
| HCl | = | hydrochloric acid |
| hpt | = | hygromycin phosphotransferase gene |
| HygR | = | hygromycin resistant |
| IPTG | = | isopropyl-thiogalactoside |
| Kb | = | kilobase |
| KCl | = | potassium chloride |
| $MgCl_2$ | = | magnesium chloride |
| mg | = | milligram |
| ml | = | millilitre |
| mm | = | millimetre |
| M | = | molar |
| ng | = | nanogram |
| nptII | = | neomycin phosphotransferase gene |
| OD | = | optical density |
| PCR | = | polymerase chain reaction |
| PEG | = | polyethylene glycol |
| RNaseA | = | ribonuclease A |
| rpm | = | revolution per minute |
| SDS | = | sodium dodecyl sulfate |
| sGFP | = | synthetic green fluorescent protein |
| Tris | = | tris (hydroxy methyl) aminomethane |
| µg | = | microgram |
| µl | = | microlitre |
| µM | = | micromolar |
| U | = | unit |
| UV | = | ultraviolet |
| vir | = | virulence |
| X-Gluc | = | 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid |

# CHAPTER I

# INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crops in the world and is the staple food for about three billion people (Toenniessen, 1996). World, and Asian, demand for rice is expected to grow in order of 2.5% per year as a result of growth in population and income per capita (World Food Council, 1991). Production and consumption are concentrated in Asia where more than 90% of all rice is produced and consumed (David, 1991).

The world total production in 1997 was 575.6 million tons produced from 936.2 million rais harvested area (Table 1.1). Of these, China and India account for about 56% of the world's production and 50% of the world's harvested area. Indonesia, Bangladesh, Vietnam and Thailand each have rice production and harvested areas greater than the total for South America.

Rice is the significant commodity for Thai's economy. It is the country's staple food, by-products of rice is also importance for human and animal consumption (Tassongchant, 1987).

Thailand is one of the world rice producers among others big sources in Southeast Asia such as China, India, Indonesia, Bangladesh and Vietnam. It has been estimated that Thailand share approximately 4% of the world rice production. It was reported that in 1997 crop, Thailand planted about 64.189 million rais of rice to produce 23.58 million tons of paddy (Table 1.2), yielding roughly 15.57 million tons of milled rice. Of this, approximately 10 million tons are used for local consumption. The balance is about 5.57 million tons left available for export. Thailand gains 65 million bahts from exporting of rice (Table 1.3).

**Table 1.1** Rice: havested area, production and yield, Thailand and selected countries, 1993-1997

| Country | Harvested area (1,000 rais) | | | | | Production (1,000 tons) | | | | | Yield per rai (kgs.) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1993 | 1994 | 1995 | 1996 | 1997 | 1993 | 1994 | 1995 | 1996 | 1997 | 1993 | 1994 | 1995 | 1996 | 1997 |
| World Total | 906,175 | 909,031 | 937,052 | 935,557 | 936,199 | 523,743 | 536,432 | 550,602 | 568,098 | 575,563 | 578 | 590 | 588 | 607 | 615 |
| Africa | 41,213 | 42,025 | 44,231 | 45,119 | 46,756 | 13,744 | 14,169 | 15,201 | 15,663 | 16,552 | 333 | 337 | 344 | 347 | 354 |
| Egypt | 3,369 | 3,619 | 3,681 | 3,688 | 4,075 | 4,161 | 4,583 | 4,788 | 4,895 | 5,585 | 1,235 | 1,266 | 1,301 | 1,327 | 1,371 |
| Nigeria | 10,475 | 10,713 | 11,225 | 11,150 | 12,800 | 2,305 | 2,427 | 2,920 | 3,122 | 3,268 | 220 | 227 | 260 | 280 | 255 |
| Madagascar | 7,556 | 7,375 | 7,188 | 7,125 | 7,375 | 2,550 | 2,360 | 2,450 | 2,500 | 2,558 | 337 | 320 | 341 | 351 | 347 |
| Others | 19,813 | 20,318 | 22,137 | 23,156 | 22,506 | 4,728 | 4,799 | 5,043 | 5,146 | 5,141 | 239 | 236 | 228 | 222 | 228 |
| NC America | 10,181 | 11,700 | 11,419 | 10,856 | 11,594 | 8,778 | 10,673 | 9,833 | 9,929 | 10,376 | 862 | 912 | 861 | 915 | 895 |
| U.S.A. | 7,169 | 8,388 | 7,825 | 7,081 | 7,675 | 7,081 | 8,971 | 7,887 | 7,771 | 8,115 | 988 | 1,070 | 1,008 | 1,097 | 1,057 |
| Dominican | 494 | 513 | 663 | 644 | 656 | 475 | 406 | 487 | 474 | 551 | 962 | 791 | 735 | 736 | 840 |
| Others | 2,518 | 2,799 | 2,931 | 3,131 | 3,263 | 1,222 | 1,296 | 1,459 | 1,684 | 1,710 | 485 | 463 | 498 | 538 | 524 |
| South America | 38,281 | 39,081 | 39,319 | 36,706 | 34,306 | 16,878 | 17,948 | 19,191 | 18,383 | 17,994 | 441 | 459 | 488 | 501 | 525 |
| Brazil | 27,569 | 27,594 | 27,338 | 24,481 | 22,481 | 10,108 | 10,499 | 11,226 | 9,990 | 9,334 | 367 | 380 | 411 | 408 | 415 |
| Colombia | 2,413 | 2,550 | 2,544 | 2,544 | 2,463 | 1,590 | 1,679 | 1,743 | 1,787 | 1,802 | 659 | 658 | 685 | 702 | 732 |
| Peru | 1,113 | 1,494 | 1,269 | 1,313 | 1,500 | 968 | 1,401 | 1,142 | 1,203 | 1,460 | 870 | 938 | 900 | 916 | 973 |
| Argentina | 875 | 881 | 1,150 | 1,206 | 1,406 | 608 | 608 | 926 | 974 | 1,208 | 695 | 690 | 805 | 808 | 859 |
| Others | 6,311 | 6,562 | 7,018 | 7,162 | 6,456 | 3,604 | 3,761 | 4,154 | 4,429 | 4,190 | 571 | 573 | 592 | 618 | 649 |
| Asia | 811,725 | 811,625 | 837,720 | 837,945 | 838,555 | 480,587 | 489,748 | 502,647 | 519,914 | 526,187 | 592 | 603 | 600 | 620 | 627 |
| China | 192,163 | 190,863 | 194,419 | 198,463 | 195,925 | 179,977 | 178,031 | 187,334 | 197,074 | 198,471 | 937 | 933 | 964 | 993 | 1,013 |
| India | 262,713 | 259,106 | 268,188 | 267,500 | 263,750 | 118,464 | 121,997 | 119,442 | 121,812 | 123,012 | 451 | 471 | 445 | 455 | 466 |
| Indonesia | 68,831 | 67,088 | 71,494 | 70,825 | 69,375 | 48,181 | 46,641 | 49,744 | 51,102 | 50,632 | 700 | 695 | 696 | 722 | 730 |
| Bangladesh | 61,338 | 61,925 | 62,200 | 62,288 | 63,606 | 27,062 | 25,248 | 25,249 | 26,531 | 28,183 | 441 | 408 | 406 | 426 | 443 |

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**Table 1.1** (continued)

| Country | Harvested area (1,000 rais) | | | | | Production (1,000 tons) | | | | | Yield per rai (kgs.) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1993 | 1994 | 1995 | 1996 | 1997 | 1993 | 1994 | 1995 | 1996 | 1997 | 1993 | 1994 | 1995 | 1996 | 1997 |
| Vietnam | 40,994 | 41,244 | 42,288 | 43,881 | 43,881 | 22,837 | 23,528 | 24,964 | 26,397 | 26,397 | 557 | 570 | 590 | 602 | 602 |
| Thailand | 53,015 | 56,095 | 56,870 | 57,920 | 61,955 | 18,447 | 21,111 | 22,016 | 22,332 | 23,580 | 348 | 376 | 387 | 386 | 381 |
| Myanmar | 34,294 | 35,894 | 37,706 | 34,656 | 37,938 | 16,760 | 18,195 | 17,670 | 17,550 | 18,900 | 489 | 507 | 469 | 506 | 498 |
| Japan | 13,369 | 13,825 | 13,238 | 12,356 | 12,206 | 9,793 | 14,976 | 13,435 | 12,930 | 12,531 | 733 | 1,083 | 1,015 | 1,046 | 1,027 |
| Philippines | 21,069 | 23,119 | 24,694 | 24,000 | 24,013 | 9,434 | 10,538 | 11,284 | 11,365 | 11,269 | 448 | 456 | 457 | 474 | 469 |
| Korea, South | 7,094 | 6,888 | 6,594 | 6,556 | 6,531 | 6,507 | 6,932 | 6,387 | 7,121 | 7,100 | 917 | 1,006 | 969 | 1,086 | 1,087 |
| Pakistan | 13,669 | 13,181 | 13,513 | 14,069 | 14,475 | 5,992 | 5,170 | 5,950 | 6,457 | 6,546 | 438 | 392 | 440 | 459 | 452 |
| Nepal | 9,088 | 8,881 | 9,356 | 9,444 | 9,444 | 3,493 | 2,928 | 3,579 | 3,711 | 3,711 | 384 | 330 | 383 | 393 | 393 |
| Cambodia | 11,400 | 9,344 | 12,025 | 12,188 | 12,188 | 2,383 | 2,223 | 3,300 | 3,390 | 3,390 | 209 | 238 | 274 | 278 | 278 |
| Sri Lanka | 5,125 | 5,606 | 5,563 | 4,125 | 4,125 | 2,570 | 2,684 | 2,810 | 2,062 | 2,610 | 501 | 479 | 505 | 500 | 633 |
| Others | 17,563 | 18,566 | 19,572 | 19,674 | 19,143 | 8,687 | 9,546 | 9,483 | 10,080 | 9,855 | 495 | 514 | 485 | 512 | 515 |
| Europe | 4,050 | 3,769 | 3,575 | 4,031 | 3,919 | 2,778 | 2,793 | 2,695 | 3,224 | 3,083 | 686 | 741 | 754 | 800 | 787 |
| Italy | 1,450 | 1,488 | 1,494 | 1,531 | 1,456 | 1,286 | 1,316 | 1,321 | 1,424 | 1,395 | 887 | 884 | 884 | 930 | 958 |
| Spain | 300 | 419 | 344 | 669 | 700 | 323 | 408 | 330 | 761 | 735 | 1,077 | 974 | 959 | 1,138 | 1,050 |
| Others | 2,300 | 1,862 | 1,737 | 1,831 | 1,763 | 1,169 | 1,069 | 1,044 | 1,039 | 953 | 508 | 574 | 601 | 567 | 541 |
| Oceania | 725 | 831 | 788 | 900 | 1,069 | 978 | 1,101 | 1,035 | 985 | 1,371 | 1,349 | 1,325 | 1,313 | 1,094 | 1,283 |
| Australia | 663 | 781 | 744 | 856 | 1,025 | 955 | 1,082 | 1,016 | 966 | 1,352 | 1,440 | 1,385 | 1,366 | 1,129 | 1,319 |
| Others | 62 | 50 | 44 | 44 | 44 | 23 | 19 | 19 | 19 | 19 | 371 | 380 | 432 | 432 | 432 |

Source : Thailand, Office of Agricultural Economics

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**Table 1.2** Rice in Thailand: (major and second rice): area, production, yield, farm price and farm value, crop year 1988/89-1997/98

| Crop year | Planted area (million rais) | Havested area (million rais) | Production (million tons) | Yield per rai (kgs.) | Farm price (Bahts per ton) | Farm value (million bahts) |
|---|---|---|---|---|---|---|
| 1988/89 | 64.677 | 61.912 | 21.263 | 343 | 3,980 | 84,626.7 |
| 1989/90 | 64.439 | 61.744 | 20.601 | 334 | 3,629 | 74,761.0 |
| 1990/91 | 61.910 | 54.949 | 17.193 | 313 | 3,608 | 62,032.3 |
| 1991/92 | 59.671 | 56.581 | 20.400 | 361 | 3,808 | 77,683.2 |
| 1992/93 | 60.453 | 57.248 | 19.917 | 348 | 3,286 | 65,447.3 |
| 1993/94 | 59.251 | 53.015 | 18.447 | 348 | 3,727 | 68,752.0 |
| 1994/95 | 60.677 | 56.095 | 21.111 | 376 | 3,857 | 81,425.1 |
| 1995/96 | 63.353 | 56.870 | 22.016 | 387 | 4,764 | 104,884.2 |
| 1996/97 | 63.728 | 57.920 | 22.332 | 386 | 5,522 | 123,317.3 |
| 1997/98 | 64.189 | 61.955 | 23.580 | 381 | 6,962 | 164,164.0 |

**Table 1.3** Production and value of milled rice exports between 1990-1997

| Year | Production (million tons) | Value (million bahts) |
|---|---|---|
| 1990 | 4.02 | 27,770 |
| 1991 | 4.17 | 30,516 |
| 1992 | 4.90 | 36,214 |
| 1993 | 4.99 | 32,947 |
| 1994 | 4.86 | 39,188 |
| 1995 | 6.20 | 48,627 |
| 1996 | 5.46 | 50,735 |
| 1997 | 5.57 | 65,088 |

Source: Office of Agricultural Economics, Ministry of Agriculture and Co-operatives.

At about 381 kg per rai, the average yield for paddy production in Thailand is not very high compared to the average yield of other major rice producing countries (602 kg/rai for Vietnam, 1,027 kg/rai for Japan, 1,057 kg/rai for USA) (Table 1.1). Cost production is also high due to the low per unit area yield. At the same time, the population has increased implying that production has to cope with the increasing requirement. Production can be increased by improvement of rice varietal characteristics and introduction of modern technologies suited to our local conditions.

## 1.1 Taxonomy of rice (*Oryza sativa* L.)

The taxonomic definition of rice is as follows (Tassongchant, 1987).

**Division** Spermatophyta
**Class** Angiospermae
**Subclass** Monocotyledonae
**Order** Graminales
**Family** Graminae
**Genus** *Oryza*
**Species** *Oryza sativa* L.
**Subspecies** *indica*
*japonica*
*javanica*

## 1.2 Rice biology

Rice (*O. sativa* L.) is a kind of short living plant related to grass. Normal life span is 3-7 months depending on variety and climate. Cultivated rice consisted of two species, *O. sativa* and *O. glaberima*. Of these, *O. sativa* is cultivated worldwide, while *O. glaberima* is grown in a few countries in West Africa (Oka, 1991).

There are 3 subspecies of *O. sativa*, *indica* (long grain), *japonica* (round grain) and *javanica* (medium grain). The *indica* rice concentrates in the warm climate belt,

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from Indochina, Thailand, India, Pakistan, Brazil and Southern U.S.A.. The *japonica* is mostly grown in cold climate countries, Japan, Korea, northern China and California. Thd *javanica* only grown in Indonesia (Oka, 1991).

Rice is harvested from field in form of paddy. Paddy is a complete seed of rice, one grain of paddy contains one rice kernel (Fig.1.1). Each paddy consists of many layers. Outermost layer is rice shells called hull. Hull consist of 2 interlocked half shells, each protects one half side of paddy. Hull consisted mostly of silica and cellulose. Next layers are called bran layers. Each layer is very thin bran film. Bran is mainly fiber, vitamin B, protein and fat, the most nutritious part of rice. At the base of each grain is embryo which will grow to actual new plant. The innermost part is rice kernel, consisting mainly starch. Rice starch consists mainly two type of starches, amylose and amylopectin. Mixture of these two starches determine cooking texture of rice (Tassongchant, 1987).

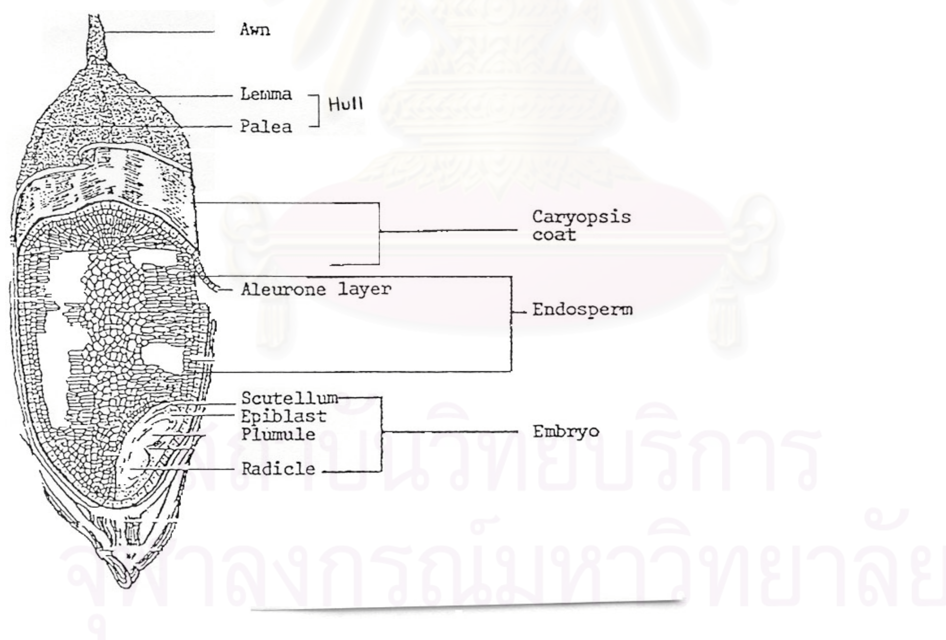



**Fig. 1.1** Longitudinal section of rice seed (Juliano, 1980)

## 1.3 *O. sativa* cv. Khao Dawk Mali 105

Khao Dawk Mali 105 (KDML 105) is a famous aromatic rice variety of Thailand and worldwide because of its aromatic, soft and tender cooked rice. KDML 105 is popularly grown under rainfed lowland in the North and Northeast of Thailand. However, KDML 105 itself is a tall variety and can not produce high enough grain yield. KDML 105 is also photoperiod sensitive which restricted its multiple cropping per year. Moreover, KDML 105 is susceptible to many insect pets and diseases, although this variety can resist to several adversed planting conditions such as moderate degree of drought salted soil or acid soil (Tassongchant, 1987).

### 1.4 Applications of biotechnology in plant breeding

Thailand is an agricultural country which produces substantial amount of food for domestic consumption and export. However, being in the tropic, agricultural production in Thailand is limited by several factors. Diseases and insect pests are the key factors affecting crop quality and yield. Chemical control has been for a long time the most common practice to cope with diseases and pests of crop plants. It is shown to become less effective due to the development of resistance among plant pests, an increasing of the cost of chemical pesticides and the adverse effect on the environment.

Conventional breeding has been used regularly for crop improvement and production of a new variety. Traditional plant breeding involves making large numbers of crosses between a range of parents which have been selected by the breeder for their desirable attributes. Some of the progeny from these crosses will show a combination of the best traits from the parents. After continued genetic recombination and a number of cycles of selection, a new variety is obtained which has a combination of desirable characteristics and which is distinct from any other variety. This process is simple in outline but complex in practice. Plant breeding involves a wide range of skills. Given this complexity it can take up to 15 years between the initial cross and the commercial release of a new variety. As a further

complication there are various limitations to plant improvement through conventional breeding. Not only are plant-breeding programmes long-term and therefore expensive, they also require the cultivation and analysis of large numbers of plants which takes both time and space. Additionally, in some cases, the breeder has limited genetic variation for incorporation into new varieties. Moreover, varieties derived from breeding program may be somewhat different from their parental lines. Horticultural characters like size and shape of fruit, color of flower, and processing quality of vegetable could be affected. Thus techiques which overcome any of these constraints are of interest and potential value to plant breeders (Connett and Barfoot, 1992).

Tissue and cell culture techniques are being explored as an innovative breeding method in the genetic modification and improvement of plants. In rice, almost parts of tissue can be used in tissue culture *in vitro* such as stem (Dun-Yi and Krikorian, 1981; Wu and Li, 1970), root (Abe and Futsuhara, 1985; Zimmy and Lorz, 1986), anther (Chu et al., 1975) and embryo (Hartke and Lorz, 1989; Boissot and Valdez, 1990). Callus induction and efficient plant regeneration for *O. sativa* L. *japonica* varieties has been reported widely (Abe and Futsuhara, 1986; Mikami and Kinoshita, 1988). However, plant regeneration from the major cultivated *indica* varieties is generally poor (Abe and Futsuhara, 1984; Kavi Kishor and Reddy, 1986). Even within the *indica* group, there are significant variations in the *in vitro* culture response among different genotypes (Peng et al., 1992).

The rapid development of biotechnology particularly plant genetic engineering offers an alternative approach for the control of plant diseases and plants pests. Genetic engineering offers an advantage over conventional breeding in a way that only one or two characters will be introduced into crop species. The overall genotypic characters of that species remain unchange.

Rice genetic engineering for insect resistance can be obtained by introducing genes conferring insecticidal activities such as endotoxin gene from *Bacillus thuringiensis* (Fujimoto et al., 1993; Cheng et al., 1998; Datta et al., 1998). Another attractive alternative is the production of proteins with insecticidal activity by the plant itself, such as plant proteinase inhibitors because they are part of plant's natural

defense system against insect predation (Duan et al., 1996; Xu et al., 1996b). Studies on developing virus resistance in rice through genetic engineering (Hayakawa et al., 1992) and improving quality of rice (Shimada et al., 1993; Burkhardt et al., 1997) have also been made. Several other genes related to agronomically important traits such as herbicide resistance (Datta et al., 1992; Christou et al., 1991), salt and water deficit (Weizhong et al., 1997; Xu et al., 1996a) and fungal resistance have also been introduced in rice (Lin et al., 1995; Stark-Lorenzen et al., 1997).

## 1.5 Gene transfer methods in plants

### 1.5.1 Vector-free gene transfer

Vector-free or dircet gene transfer systems introduced foreign DNA as a naked molecule without a biological vector. There are broadly three approaches to direct gene transfer, which can be described as chemical, electrical and physical. Each is designed to overcome the barrier to DNA uptake that is presented by the cell wall and the plasma membrane. The plant cell wall is a densely structured organelle in which the cellulose microfibrils are intercalated with pectins, hemicelluloses and proteins, which together can prevent the diffusion of large molecules, such as nucleic acids. Furthermore, since DNA is a charged and hydrophilic molecule it does not move freely through the lipid bilayer of the plasma membrane. For these reasons it has been considered advantageous to develop direct gene transfer systems that utilize protoplasts: cells that have had the cell walls removed by enzymatic digestion.

#### 1.5.1.1 Polyethylene glycol (PEG) treatment

The PEG method involves in plasma-membrane destabilizing agent. This type of chemical transformation uses mostly protoplasts that are simply incubated with DNA in buffers containing PEG (Weising et al., 1988). The exact mechanism of action of these diverse agents is not known, but is regarded as destabilizing membrane structures (Kahl and Weising, 1993). However, the production of transgenic plants with PEG treatment was often restricted to certain plants in which the regeneration

system from protoplasts is well established. Moreover, this method suffers from many limitations. The choice of explant for protoplast isolation is often restricted to embryogenic cell suspension. It is very difficult to initiate and maintain these cultures and the regeneration capacity of these cultures has been shown to decline gradually with increasing age of the cultures (Jähne et al., 1995). Further, plant regeneration from protoplasts is labor-intensive, inefficient, time consuming, and is strongly genotype dependent.

### 1.5.1.2 Electroporation

The use of electroporation for gene delivery has been preferred over PEG because it was found to be more efficient (Zhang et al., 1988). Electroporation is the application of high-voltage electric pulses to cells to induce transient membrane pores, allowing entry of macromolecules including DNA (Rathus and Birch, 1991) However, this method also suffers from the same drawbacks as the PEG method because this also relies mainly on the use of protoplasts for the introduction of foreign genes.

### 1.5.1.3 Microprojectile bombardment or particle acceleration

The microprojectile bombardment system as a method of gene delivery in intact cells and tissues has been enthusiastically employed in cereals soon after its development (Christou, 1997). This method is based on high velocity bombardment of plant cells with DNA-coated microprojectiles (tungsten or gold) accelerated by gun powder discharge or pressurized helium or electric current (Sanford, 1990). This method has been widely used in the production of transgenic plants. The reason for the popularity of this method is that transgenic plants can be obtained following bombardment of any regenerable tissue, thereby eliminating the requirement of isolation and regeneration of protoplasts (Cao et al., 1991). Like other methods, the particle acceleration too has its drawbacks. In some cases, the copy number and rearrangement of the introduced DNA is high, thereby rendering transgene prone to gene silencing and causing genomic changes (Bover et al., 1996). The other major

drawback is the restricted availability of the equipment because of its high cost (Christou and Ford, 1995).

### 1.5.2 *Agrobacterium*-mediated transfer of foreign genes into target plants

*A. tumefaciens* is exploited by many plant biologists in molecular and genetic studies to introduce DNA into plants. Although best known for this practical application, the transfer of DNA from bacterium to plant comprises fundamental biological processes, many of which are largely uncharacterized.

*Agrobacterium* carries three genetic components required for plant cell transformation. Two of these, the T-DNA and the virulence (*vir*) region, are located on the large (roughly 200 kb) Ti plasmid (Fig. 1.2) (Binns and Thomashow, 1988). The T-DNA is the DNA segment that is transferred from *Agrobacterium* to the plant cell. The *vir* region is organized into six complementation groups that are either absolutely essential for (*virA*, *virB*, *virD* and *virG*) or that enhance the efficiency of (*virC* and *virE*) plant cell transformation. The third bacterial component of the T-DNA transfer process resides in the *Agrobacterium* chromosome. Three chromosomal virulence loci, *chvA* and *chvB* and *pscA*, encode products involved in the binding of *Agrobacterium* to plant cells during the infection process (Zambryski, 1988).

During infection by *Agrobacterium*, a piece of DNA is transferred from the bacterium to the plant cell (Fig. 1.2). The piece of DNA is a copy of a segment called the T-DNA (transferred DNA). It is carried on a specific plasmid, the Ti-plasmid (tumor-inducing). The T-DNA is delimited by 25-bp direct repeats that flank the T-DNA. Any DNA between these borders will be transferred to a plant cell (Walden, 1993). Wild-type T-DNA encode enzymes for the synthesis of the plant growth regulators; auxin and cytokinin, and the production of these compounds in transformed plant cells results in the tumorous phenotype. In addition, wild-type T-DNA also encodes enzymes for the synthesis of novel amino acid derivatives called opines. The Ti-plasmid encodes enzymes for their catabolism; hence, *Agrobacterium* has evolved to genetically commandeer plant cells and use them to produce

compounds that they uniquely can utilize as a carbon / nitrogen source (Kahl and Weising, 1993).

The processing and transfer of T-DNA are mediated by products encoded by the *vir* (virulence) region, which is also resident on the Ti-plasmid (Stachel and Nester, 1986). Those *vir* genes, whose products are dirctly involved in T-DNA processing and transfer, are tightly regulated so that expression occurs only in the presence of wounded plant cells, the targets of infection. Control of gene expression is mediated by the VirA and VirG proteins, a two-component regulatory system. VirA detects the small phenolic compounds released by wounded plants resulting in autophosphorylation (Fig 1.2, step 1). VirA phosphoryloation of VirG then leads to activation of *vir* gene transcription (Winans, 1992).

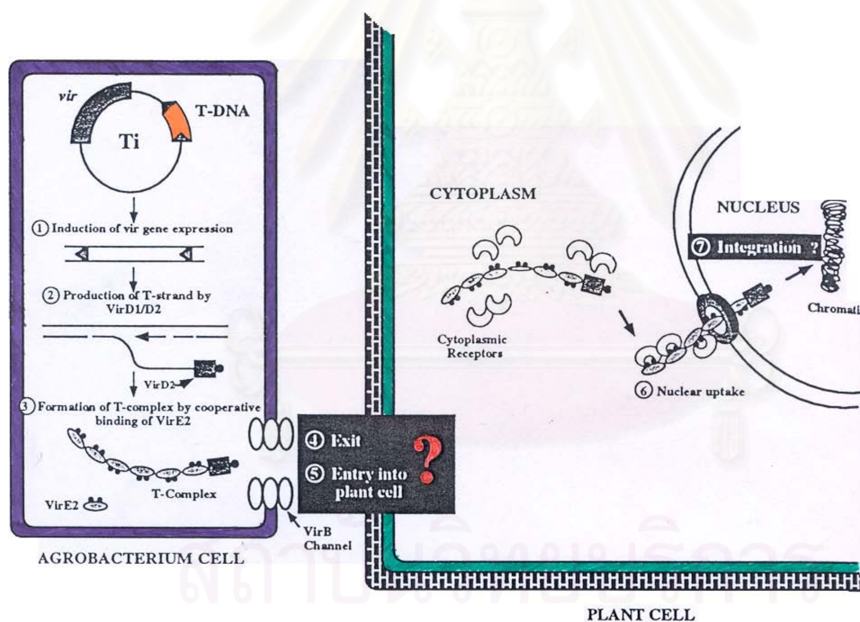



**Fig. 1.2** Basic steps in the transformation of plant cells by *A. tumefaciens* (Zupan and Zambryski, 1995).

Following *vir* gene induction, the production of a transfer intermediate begins with the generation of the T-strand, an single strand copy of the T-DNA (Stachel et al, 1986). VirD1 and VirD2 are essential for this process (Filichkin and Gelvin, 1993). Together, VirD1 / VirD2 recognize the 25-bp border sequence and produce an single strand endonucleolytic cleavage in the bottom strand of each border (Fig 1.2, step 2). These nicks are used as the initiation and termination sites for T-strand production. After nicking, VirD2 remains tightly associated with the $5^{/}$ end of the T-strand. The lone VirD2 at the $5^{/}$ end gives the T-complex a polar character that may ensure that, in subsequent steps, the $5^{/}$ end is the leading end. T-strand production is thought to result from the displacement of the bottom strand of the T-DNA between the nicks (Zupan and Zambryski, 1995).

The T-strand must travel through numerous membranes and cellular spaces before arrival in the plant nucleus. Thus, to preserve its integrity, it was hypothesized that the T-strand likely travels as an single strand DNA-protein complex. VirE2 is an inducible single strand nucleic acid-binding protein encoded by the *vir*E locus that binds without sequence specificity. VirE2 binds tightly and cooperatively, which means that a T-strand would be completely coated (Fig. 1.2, step 3). Consequently, degradation by nucleases would be prevented and, indeed, *in vitro* binding of VirE2 renders single strand DNA resistant to nucleolytic degradation. Finally, binding of VirE2 unfolds and extends single strand DNA to a narrow diameter of 2 nm, which may facilitate transfer through membrane channels. The T-strand along with VirD2 and VirE2 are termed the T-complex (Zupan and Zambryski, 1995).

Subsequently, the T-complex must exit the bacterial cell (Fig. 1, step 4) passing through the inner and outer membranes as well as the bacterial cell wall. It must then cross the plant cell wall and membrane (Fig. 1, step 5). Once inside the plant cell, The T-complex targets to the plant cell nucleus and crosses the nuclear membrane (Fig. 1, step 6), after which the T-strand becomes intergrated into a plant chromosome (Fig. 1, step 7). In the context of experimental chronology and relevant results, step 1 through 3 (Fig. 1) have been studied. Current and recent research has related to step 4 and 6. Entering the plant cell (step 5) and the mechanics of

integration (step 7) are almost completely uncharacterized (Zupan and Zambryski, 1995).

## 1.6 Selectable marker genes

Selectable markers are used to select for the specific growth of transformed cells amongst a background of non-transformed individuals. This is important because transformation frequencies remain normally relatively low. Such markers allow growth, or at least viability, in the presence of the selective agent. Routinely, resistance to antibiotics or phytotoxins such as herbicides has been used. In the former case, antibiotic resistance genes derived from bacteria have been utilised (e.g. kanamycin and hygromycin), whereas in the latter case genes encoding products which are more tolerant to herbicides, for example glyphosate and phosphinothricin have been used (Walden, 1993).

### 1.6.1 Neomycin phosphotransferase

The most commonly used selectable gene is *npt*II. It was isolated from transposon Tn 5 of an *E. coli* strain. This gene codes for the enzyme neomycin phosphotransferase that detoxifies several aminoglycoside antibiotics such as kanamycin, geneticin (G418) and neomycin. However, kanamycin can be used as a selective agent during regeneration of protoplasts but it is not effective for selection of transformed calli. In addition, many calli recovered after kanamycin selection are unable to regenerate green plants (Ayres et al., 1994; Toriyama et al., 1988).

### 1.6.2 Hygromycin phosphotransferase

The gene *hpt* (or *hph*) was isolated from *E. coli*. It codes for the enzyme hygromycin phosphotransferase. This gene therefore causes resistance to the antibiotic compound hygromycin. The *hpt* / hygromycin B combination was successfully employed in the genetic transformations of tobacco, *Arabidopsis*, maize and rice (Schrott et al., 1995). Hygromycin is a more potent phytotoxic compound

than kanamycin; especially in cereal crops (Galun and Breiman, 1997). Hygromycin allows clear discrimination between transformed and non-transformed tissues and problems with albinos or the ferility of regenerants have not been reported (Ayres et al., 1994).

### 1.6.3 Phosphinothricin acetyltransferase (*bar* or *pat*)

Two bacterial genes (*bar* from *Streptomyces hygroscopicus* and *pat* from *S. viridochromogenes*) have the ability to detoxify phosphinothricin (PPT) by acetylation. These genes code for phosphinothricin acetyltransferase which confers resistance to phosphinothricin (D'Halluin et al., 1992). Phosphinothricin which is a structural analog of glutamine, inhibits the glutamine synthase (GS) of both plant and bacterial origin. Inactivation of the GS will lead to ammonia accumulation which is toxic to the cell. There are derivatives of this compound that are also used as herbicides (e.g. Bialaphos, Basta).

## 1.7 Reporter genes in plants

Reporter genes are coding sequences that, upon expression in the transgenic plant, provide a clear indication that genetic transformation did take place (Galun and Breiman, 1997). In general, reporter genes should have the following characteristics: 1) the genetic organization should be well described, 2) the gene products should not be present in the organism or tissue under study, 3) the gene products should be well characterized with regard to biochemical activity, 4) substrate dependence and stability, and 5) the product of the reaction catalyzed by the reporter gene product should be stable, easily detectable, and quantifiable (Crazzolara et al., 1995). The four most widely used systems of reporter genes are chloramphenicol acetyltransferase (CAT), luciferase, β-glucuronidase (GUS) and Green fluorescent protein (GFP).

### 1.7.1 Chloramphenicol acetyltransferase (CAT)

CAT was the first reporter gene used to monitor transcriptional activity in cells (Bronstein et al., 1994). CAT is a bacterial enzyme that can detoxify chloramphenicol, an inhibitor of prokaryotic protein synthesis, by catalysing the transfer of acetyl groups from acetyl CoA to the 3-hydroxyl position of chloramphenicol. However, the assay relies on radiochemicals, and sensitivity of the assay are not as broad as for other reporters (Bronstein et al., 1994).

### 1.7.2 Luciferase (LUX)

The luciferase reporter gene system is based on a luminescence reaction. It mimics the *in vivo* reaction that take place in certain insects (fireflies) and bacteria by enzyme, luciferin 4-monooxygenase. It can be used for *in vitro* assays, in the presence of the substrate, luciferin supplemented by ATP, Mg++ and molecular oxygen. The reaction yields a yellow-green (560 nm) light (Galun and Breiman, 1997). Although, luciferase can be monitored *in vivo* (Ow et al., 1986) but requires an exogenous substrate and emits light only at very low intensity (Ow et al., 1986).

### 1.7.3 β-glucuronidase (GUS)

GUS is encoded by the *Escherichia coli uidA* gene (Jefferson et al., 1986, 1987) and has become the most widely used reporter gene in plants. The protein has a molecular weight of 68.2 kDa. The best substrate currently available for histochemical localization of β-glucuronidase activity in tissues and cells is 5-bromo-4-chloro-3- indolyl glucuronide (X-Gluc). The product of glucuronidase activity on X-Gluc is colorless. Instead, the indoxyl derivative produced must undergo an oxidative dimerization to form the insoluble and highly colored indigo dye. This dimerization is stimulated by atmospheric oxygen, and can be enhanced by using an oxidation catalyst such as a potassium ferricyanide/ferrocyanide mixture. It is the efficient reporter that can be used to locate its expression in plant tissues and organs without the need to extract the respective tissue. However, the substrate for dectection of GUS activity (X-Gluc) is expensive and the X-Gluc-stained plant material is killed

by the respective GUS assay; it is therefore not a vital staining (Jefferson et al., 1986, 1987).

### 1.7.4 Green fluorescent protein (GFP)

Green fluorescent proteins are a unique class of proteins also involved in the bioluminescence of many jellyfish (Gilroy, 1997). The GFP from *Aequorea victoria* is a 26.9-kDa protein (238 amino acid) that fluoresces green upon excitation with blue light (Prasher et al., 1992). Native GFP from *A. victoria* absorbs optimally at 395 nm (with a weaker absorbance at 470 nm) and emits at 509 nm (Gilroy, 1997).

The structure of GFP has been solved using seleniomethionyl-substituted protein and multi-wavelength anomalous dispersion (MAD) phasing methods. The electron density maps produced by the MAD phasing were very clear, revealing a dimer comprised of two quite regular-barrels with 11 strands on the outside of cylinders (Fig 1.3). The fluorophore is highly protected, located on the central helix within a couple of Angstroms of the geometric center of the cylinder (Yang et al., 1996).

*Aequorea* GFP owes its visible absorbance and fluorescence to a *p*-hydroxybenzylideneimidazolinone chromophore (Prasher et al., 1992) formed by cyclization of Ser65, Tyr66 and Gly67 and 1,2-dehydrogenation of the tyrosine. This mechanism occurred in post-translational modification. Studies of recombinant GFP expression in *E.coli* led to a proposed sequential mechanism initiated by a rapid cyclization between Ser65 and Gly67 to form a imidazolin-5-one intermediate followed by a much slower (hours) rate-limiting oxygenation of the Tyr66 side chain by $O_2$ (Fig. 1.4) (Cubitt et al., 1995). Combinatorial mutagenesis suggests that the Gly67 is required for formation of the fluorophore (Delagrave et al., 1995). While no known co-factors or enzymatic components are required for this apparently auto-catalytic process.

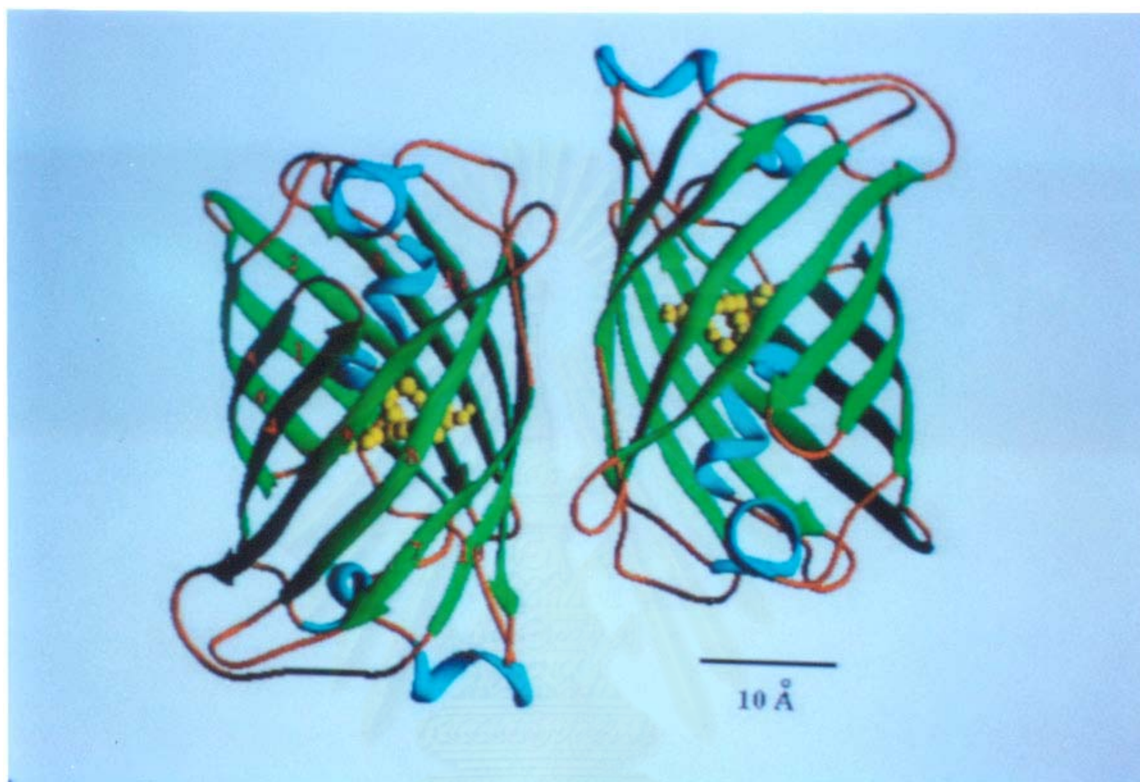



**Fig. 1.3** The overall shape of the green fluorescent protein and its association into dimers. Eleven strands of β-sheet (green) form the walls of a cylinder. Short segments of α-helices (blue) cap the top and bottom of the 'β-can' and also provide a scaffold for the fluorophore which is near geometric center of the can. This folding motif, with β-sheet outside and helix inside, represents a new class of proteins. Two monomers are associated into a dimer in the crystal and in solution at low ionic strengths. This view is directly down the two-fold axis of the non-crystallographic symmetry (Yang et al., 1996).

GFP has been used extensively in plant systems, in localization studies and as a screenable marker for gene transfer (Kohler et al., 1997). Some plant species (e.g. *Arabidopsis*) show little or no expression of GFP fluorescence as a result of aberrant splicing of the GFP message (Haseloff et al., 1997). Modification of GFP to remove a cryptic intron site (a sequence recognised as an intron in *Arabidopsis*, leading to aberrant mRNA) resulted in successful detection of GFP fluorescence (Haseloff et al., 1997). Improvements in GFP to enhance its use in plants, including fluorescent signal and codon usage, have been made by several groups (Pang et al., 1996). Chiu et al. (1996), using the S65T mutant, showed 20-fold higher expression in maize leaf cells, and detected GFP driven by weak promoters in a broad range of plant hosts.

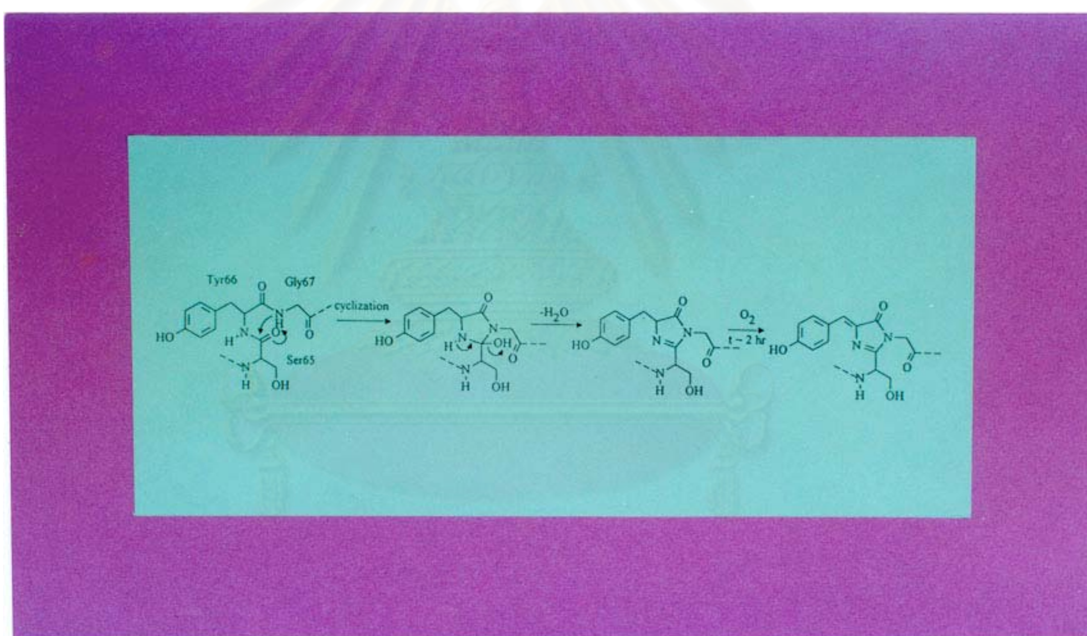



**Fig.1.4** Proposed biosynthetic mechanism for the green fluorescent protein (GFP) chromophore, in which cyclization precedes oxidation (Cubitt et al., 1995).

By combining several modifications one can produce highly optimized versions of GFP that lead to cumulative gains in GFP expression and utility. Haseloff et al. (1997) combined several changes in GFP that improve its function. Modification of codon usage ensures proper mRNA processing and removal of the cryptic intron. Amino acid substitutions for improved thermostability, folding, enhanced fluorescence, and altered spectral properties are useful for work in plants and in targeting to the endoplasmic reticulum. The use of GFP has the potential to improve monitoring of both gene transfer and expression in plants, especially with the ease (no addition of substrate, easy assay) and noninvasive nature of the assay.

GFP can function as a protein tag, as it tolerates N- and C- terminal fusion to a broad variety of proteins many of which have been shown to retain native function (Moores et al., 1996, Cubitt et al., 1995). When expressed in mammalian cells fluorescence from wild type GFP is typically distributed throughout the cytoplasm and nucleus, but excluded from the nucleolus and vesicular organelles (Cubitt et al., 1995). However, highly specific intracellular localization including the nucleus, mitochondria (Rizzuto et al., 1996), secretory pathway (Kaether et al., 1995), plasma membrane (Marshall et al., 1995) and cytoskeleton (Kahana et al., 1995) can be achieved via fusions both to whole proteins and individual targeting sequences. The enormous flexibility as a noninvasive marker in living cells allows for numerous other applications such as a cell lineage tracer, reporter of gene expression and as a potential measure of protein-protein interactions (Mitra et al., 1996).

GFP has attracted widespread interest and is considered to have several advantages over other visual marker genes. First, the fluorescence emission of GFP does not require a cofactor or a substrate; fluorescence results from an internal *p*-hydroxybenzylideneimidazolinone chromophore generated by cyclization and oxidation of a Ser-Tyr-Gly sequence at amino acid residues 65 to 67 (Cody et al., 1993). Detection of GFP in living cells thus only requires excitaion by light at 395 or 470 nm. In contrast, the assay of GUS (Jefferson et al., 1987) expression is cytotoxic and firefly luciferase (Ow et al., 1986; Millar et al., 1995) requires an exogenous substrate (luciferin) for detection.

The second advantage of GFP is that it is relatively small (26.9-kDa) and can tolerate both N- and C- terminal protein fusions, lending itself to studies of protein localization (Wang and Hazelrigg, 1994). Another advantage of GFP is that GFP mutants with shifted wavelengths of absorption and emission have been isolated (Heim et al., 1994 and 1995), which permits simultaneous use and detection of multiple reporter genes. In addition, some GFP mutants exhibit a more rapid formation of the chromophore and higher excitation peaks at 475 to 490 nm than does the wild-type GFP protein, which results in increased detection sensitivity (Heim et al., 1995).

Modified versions have been utilized to a greater extent in maize (Chiu et al., 1996 and Reichel et al., 1996), alfalfa protoplasts (Reichel et al., 1996), the cells of *Arabidopsis* (Haseloff and Amos, 1995; Haseloff et al., 1997; Pang et al., 1996 and Sheen et al., 1995), corn (Pang et al., 1996), rice (Yue et al., 1997), soybean (Plutz et al., 1996), pine (Tian et al., 1997), sugarcane (Elliott et al., 1998 and 1999) and barley (Ahlandsberg et al., 1999). It is conceivable that GFP can be used in place of selectable markers, such as those conferring antibiotic or herbicide resistance to recover transformed cells, tissues, organs and ultimately plants. For example, in transformation systems in which *in vivo* technology is used, it would be much more cost-efficient to use an *in vivo* marker such as GFP rather than GUS. Chimeric transformants can be identified easily and nondestructively. Thus, GFP may reduce the cost of transformation and make transformaion systems of economically less impotant crops, model and wild plants more feasible.

### 1.8 Rice transformation using *Agrobacterium*

Systems of *Agrobacterium*-mediated transformation have been well established for many dicotyledonous plants. However, monocotyledonous plants, in particular cereal plants, were originally outside the host range of *A. tumefaciens* (Bevan, 1984 and De Cleen and De Ley, 1985). Thus, *Agrobacterium*-mediated gene transfer has been less successful in cereals and other monocots.

In recent years, some examples on the transformation of monocots using *Agrobacterium* were presented, for example, in *Asparagus officinalis* (Bytebier et al., 1987), *Zea mays* (Citovsky et al., 1994). In 1990, Raineri et al., described the production of transformed cells of the *japonica* cultivar (cv. Nipponbare and cv. Fujisaka 5) by co-cultivatation of mature embryos with *Agrobacterium* containing the GUS and *npt*II genes. Results of Southern blotting indicated the integration of the T-DNA and then expressed in rice cells but no transgenic plants were regenerated.

Chan et al. (1993) transformed immature *japonica* rice embryos (10-12 day after pollination) (cv.Tainung 62) with *A. tumefaciens* A281 containing the GUS and *npt*II genes. The phenolic compounds from a potato suspension culture appeared to improve transformation efficiency. Transformation and integration of T-DNA into genomic DNA was confirmed by Southern blot analysis of the progeny. Transformation frequencies of 6.8% were obtained. Contamination by *A. tumefaciens* in the transgenic plants was ruled out by stripping the Southern blot and rehybridizing with a probe for *vir*B DNA.

Hiei et al., (1994) subsequently reported a method for efficient production of transgenic rice plants from calli of *japonica* cultivars (cv. Tsukinohikari, cv. Asanohikari and cv. Kosihikari) that had been co-cultivated with *A. tumefaciens* containing *npt*II, *hpt* and GUS genes. Addition of acetosyringone (100 μM) in the *Agrobacterium* suspension and co-culture media proved to be indispensable for successful transformation. A large number of morphologically normal, fertile, transgenic rice plants were obtained by co-cultivation of rice tissues with *A. tumefaciens*. The efficiency of transformation was between 12.8 and 28.6% similar to that obtained by the methods used routinely for transformation of dicotyledons with the bacterium. Stable integration, expression and inheritance of transgenes were demonstrated by molecular and genetic analysis of transformants in the R0, R1 and R2 generations. Sequence analysis revealed that the boundaries of the T-DNA in transgenic rice plants were essentially identical to those in transgenic dicotyledons.

In 1996, Rashid et al. developed the reproducible system for the production of transgenic plants in *indica* rice using *Agrobacterium*-mediated gene transfer. Three-

week-old scutella calli served as an excellent starting material. These were infected with an *A. tumefaciens* strain EHA101 carrying *hpt* and GUS genes. Inclusion of acetosyringone (50 μM) in the *Agrobacterium* suspension and co-culture media proved to be indispensable for successful transformation. Transformation efficiency of Basmati 370 was 22% which was as high as reported in *japonica* rice and dicots. A large number of morphologically normal, fertile transgenic plants were obtained. Integration of foreign genes into the genome of transgenic plants was confirmed by Southern blot analysis. GUS and *hpt* genes were inherited and expressed in R1 progeny. Mendelian segregation was observed in some R1 progeny.

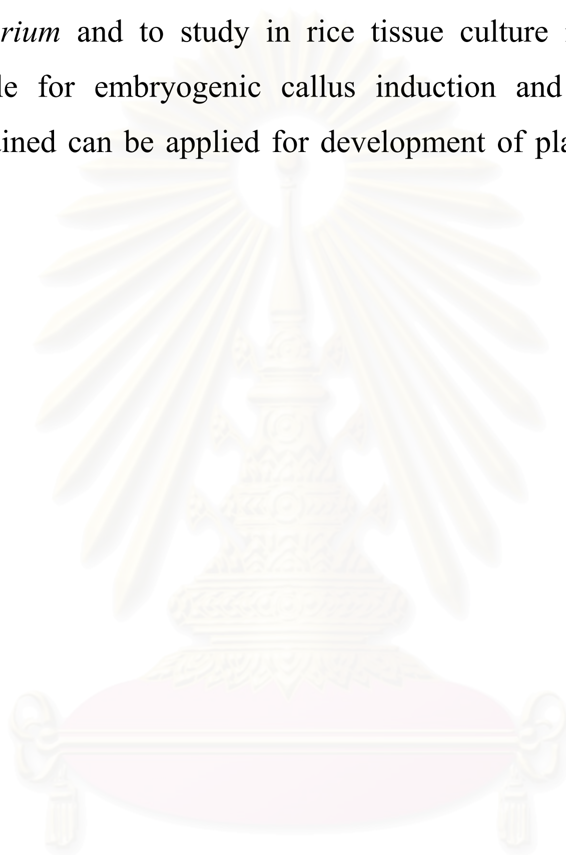
Cheng et al., (1998) transformed rice in nine cultivars with fully modified (plant codon optimized) versions of two synthetic *cryIA (b)* and *cryIA (c)* coding sequences from *Bacillus thuringiensis* as well as the *hpt* and GUS genes by co-cultivation with *A. tumefaciens* LBA4404 and EHA105. The integration, expression and inheritance of these genes were demonstrated in R0 and R1 generations by Southern, Northern and Western analysis.

Ku et al., (1999) used an *Agrobacterium*-mediated transformation system to introduce the intact gene of maize phosphoenolpyruvate carboxylase (PEPC), which catalyzes the initial fixation of atmospheric $CO_2$ in $C_4$ plants into the $C_3$ crop rice. Most transgenic rice plants showed high-level expression of the maize gene; the activities of PEPC in leaves of some transgenic plants were two-to threefold higher than those in maize, and the enzyme accounted for up to 12% of the total leaf soluble protein. RNA gel blot and Southern blot analyses showed that the level of expression of the maize PEPC in transgenic rice plants correlated with the amount of transcript and the copy number of the inserted maize gene. Physiologically, the transgenic plants exhibited reduced $O_2$ inhibition of photosynthesis and photosynthetic rates comparable to those of untransformed plants. The results demonstrate a successful strategy for installing the key biochemical component of the $C_4$ pathway of photosynthesis in $C_3$ plants.

Among rice, *indica* varities are considered recalcitrant to tissue culture and genetic manipulation (Hiei et al., 1997). The genetic transformation of *indica* rice

employing *Agrobacterium*, however, has been restricted to a few reports. In this study, the KDML 105 cultivars, which comprise an important group of *indica* rice, was transformed using *Agrobacterium*.

The objective of this thesis is to create the transformed rice (*O. sativa* cv. KDML 105) with green fluorescent protein (GFP) reporter gene by co-cultivation with *Agrobacterium* and to study in rice tissue culture including composition of medium suitable for embryogenic callus induction and plant regeneration. The knowledge obtained can be applied for development of plant genetic engineering in Thailand.



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# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Equipments and reagents

### 2.1.1 Instruments

- Autoclave LS-2D (Rexall industries Co. Ltd., Taiwan)
- Automatic micropipettes P10, P20, P100, P200 and P1000 (Gilson Medical Electrical S.A., France)
- A –20 $^{o}$C Freezer
- A –80 $^{o}$C Freezer
- Filter paper (Whatman No. 1) (Whatman International Ltd., England)
- Fluorescent microscope BX50 (Olympus Optical Co., Ltd., Japan)
- Gene pulser (Bio-Rad Laboratories, USA)
- Heating block Bd 1761G-26 (Sybron Thermermolyne Co., USA)
- Incubator BM-600 (Memmert GambH, Germany)
- Longwave UV Lamp 365 nm (Schleicher & Schuell, Germany)
- Microcentrifuge tube 0.5 and 1.5 ml (Bio-RAD Laboratories, USA)
- PCR Thermal cycler : model 2400 (Perkin Elmer)
- PCR Thin wall microcentrifuge tube 0.2 ml (Perkin Elmer)
- PCR Workstation : Model # P-036 (Scientific Co., USA)
- Pipette tips 10, 20, 100 and 1000 μl (Bio-RAD Laboratories, USA)
- Power supply : Power PAC 300 (Bio-RAD Laboratories, USA)
- Refrigerated microcentrifuge : Kubota 1300 (Kubota, Japan)
- Refrigerated centrifuge : Model J-21C (Beckman Instrument Inc., Japan)
- Spectrophotometer DU 650 (Beckman, USA)
- White/UV Transilluminator : UVP Image Store 7500 (Mitshubichi Electric Corporation, Japan)

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### 2.1.2 Chemicals

- Absolute ethanol (Merck, Germany)
- Acetosyringone ((Fluka, Switzerland)
- Agarose (FMC BioProducts, USA)
- Bacto-agar (Difco, USA)
- Bacto-yeast extract (Difco, USA)
- Bacto-tryptone (Difco, USA)
- Boric acid (Merck, Germany)
- 5-Bromo-4-chloro-3-indolyl-β -D-glucuronic acid (X-Gluc) (CLONTECH Laboratories Inc., USA)
- Bromphenol blue (Merck, Germany)
- Cetyltrimethylammonium bromide (CTAB) (Sigma Chemical Co., USA)
- Chloroform (Merck, Germany)
- Ethidium bromide (Sigma Chemical Co., USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)
- 100 mM dATP, dCTP, dGTP, dTTP (Promega Corporation Medison, Wisconsin)
- GeneAmp PCR core reagents (Perkin Elmer Cetus, USA)
  : 10x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl)
  : 25 mM $MgCl_2$
- Isoamyl alcohol (Merck, Germany)
- Phenol, crystal (Fluka, Germany)
- Sodium acetate (Merck, Germany)
- Sodium dodecyl sulfate (Sigma Chemical Co., USA)
- Sodium hydroxide (Merck, Germany)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)

### 2.1.3 Antibiotics

- Cefotaxime (Fluka, Switzerland)
- Hygromycin B (Sigma Chemical Co., USA)
- Kanamycin (Sigma Chemical Co., USA)
- Rifampicin (Sigma Chemical Co., USA)
- Tetracyclin (Sigma Chemical Co., USA)

### 2.1.4 Kit

- QIAquick Gel Extraction kit (QIAGEN, Germany)

### 2.1.5 Enzymes

- Ampli *Taq* DNA polymerase (Perkin Elmer Cetus, USA)
- *Eco*RI (New England BioLabs, USA)
- *Hin*dIII (New England BioLabs, USA)
- Lysozyme (Sigma Chemical Co., USA)
- RNase A (Sigma Chemical Co., USA)
- T4 DNA ligase (Pharmacia, USA)

### 2.1.6 Bacterial strains

- *Escherichia coli* strain XL1 Blue
  ($F^{/}$:: Tn*10 proA*$^{+}$*B*$^{+}$*lacI*$^{q}$ *Δ(lacZ)M15/recA1 endA1 gyrA96(Nal*$^{r}$*)*
  *thi* *hsdR17* ($r_k^{-}$ $m_k^{+}$) *supE44 relA1 lac*)

- *Agrobacterium tumefaciens* strain EHA105 (pEHA105); a hypervirulent, L,L-succinamopine helper strain. (pEHA105 is a T-DNA deletion derivative of pTiBo542, the hypervirulent Ti plasmid of *A. tumefaciens* strain A281) (Hood et al., 1993).

### 2.1.7 Binary vectors

- pCAMBIA5305 (Appendix C)
- pCAMBIA1301 (Appendix C)

## 2.2 Rice tissue culture

One basic premise to obtain transformed rice plant is to define the *in vitro* culture conditions necessary for regeneration of rice cultivars then the tests had been performed to determine the proper condition and most suitable ingredient of medium for culturing rice variety.

### 2.2.1 Production of embryogenic calli from mature embryos

The *indica* rice cultivar (*Oryza sativa* L.) KDML 105 was obtained from the Bangkhen rice research center. Mature seeds were dehulled and first sterilized with 70% ethanol for 1 minute and then with 2.5% sodium hypochlorite for 40 minutes with shaking. The seeds were further rinsed 3 times with sterilize deionized water. These were cultured on 2NB (Li et al., 1993) and 2MS (Murashige and Skoog, 1962) medium (Appendix A) supplemented with 2 mg/l of 2,4-dinitrophenoxy acetic acid (2,4-D) for callus induction. The cultures were incubated in the dark at 28 $^{o}$C for 6 weeks. After 6 weeks, the percentage of seeds producing embryogenic calli were recorded. The embryogenic calli observed as being compact, yellowish and granular (Peterson and Smith, 1991) were separated with sterile scalpel and subcultured on fresh medium. Actively growing embryogenic calli (1-2 mm in diameter) were used for plant regeneration and transformation experiments.

### 2.2.2 Regeneration of embryogenic calli

Two-month-old embryogenic calli having been subcultured every week were used in regeneration. Embryogenic calli were transferred to four different regeneration media varying growth regulator concentrations; MS1-RE, NB1-RE, NB2-RE and NB4-RE (Appendix A). The cultures were incubated at 28 $^{o}$C under a 16/8 hours light/dark photoperiod. After 4 weeks, the number of embryogenic calli produced shoot, green spot and root were counted and the percentage of these were calculated.

## 2.3 Transformation vectors

The transformation vector, pCAMBIA5305, contains the synthetic green fluorescent protein (sGFP) with optimal human codons as a reporter gene, the hygromycin-resistant gene (*hpt*) with an intron as a plant selectable marker within the left and right border of T-DNA (Appendix C). Each gene was under the control of an 35S promotor from cauliflower masaic virus (CaMV). The *npt*II gene encoding resistance to kanamycin was used as a bacterial selectable marker.

The pCAMBIA1301 contains the GUS (*uid*A) with an intron as a reporter gene, the hygromycin-resistant gene (*hpt*) as a plant selectable marker within the T-DNA. Each gene was under the control of an 35S promotor. The *npt*II gene encoding resistance to kanamycin was used as a bacterial selectable marker. This vector habours the pUC18 polylinker within the *lac*Zα fragment allowed blue/white screening of clones in *E. coli* cloning work.

These transformation vectors have a wide-host-range origin of replication from the *Pseudomonas* plasmid pVS1; the pBR322 origin (pMB9-type) to allow high-yielding DNA preparations in *E. coli* and the T-DNA borders. Intron from castor bean catalase eliminates any possibility of read through or inappropriate GUS and *hpt* production in prokaryotes, such as *Agrobacterium*. This intron is, however, efficiently spliced in dicots and monocots.

### 2.3.1 Combination of GFP and GUS reporter gene

#### 2.3.1.1 Preparation of GFP fragment

##### 2.3.1.1.1 Digestion of pCAMBIA5305 with *Hin*dIII and *Eco*RI

Five micrograms of pCAMBIA5305 were digested with 5 units of *Hin*dIII and *Eco*RI in the 100 µl reaction mixture using the condition recommended by the manufacturer. The reaction was incubated at 37 $^{o}$C overnight. A portion of the sample was analyzed for the completion of digestion by agarose gel electrophoresis.

##### 2.3.1.1.2 Agarose gel electrophoresis

Agarose gel electrophoresis is the standard method used for separation DNA fragments on the basis of their molecular weight and used for rough estimation of DNA on the basis of its direct relationship between the amount of DNA and the level of the fluorescence after ethidium bromide staining. The DNA was run on 0.7% agarose gel in 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA, pH 8.3). The gel was prepared by adding 0.35 g of agarose to 50 ml of 1X TBE buffer. Agarose was solubilized by heating in a microwave oven and then allowed to cool to 50-60 $^{o}$C before pouring into a plastic gel former with a preset well-forming comb. Ten microlitres of digested DNA sample were mixed with 2 µl of the loading dye (0.25% bromophenol blue and 25% Ficoll 400) before loading into the well of gel which was submerged in the TBE buffer. The 100 bp, 200 bp DNA ladders and $\lambda$ / *Hin*dIII were used as standard DNA markers. Electrophoresis was operated at 5 volts/cm until bromophenol blue moved to approximately 0.5 cm from the bottom of the gel. The electrophoresed gel was stained with a 2.5 µg/ml ethidium bromide for 5 minutes and subsequently destained in an appropriate amount of water with gently shaking for 10 minutes to remove unbound ethidium bromide from agarose gels. The DNA fragments were visualized as fluorescent bands under a UV transilluminator and photographed through a red filter with Kodak Tri-X-Pan 400 film.

### 2.3.1.1.2 DNA fragment elution

The 1.5 kb fragment was recovered from the agarose gel by using QIAquick Gel Extraction Kit (QIAGEN, Germany). After electrophoresis, the desired DNA fragment was excised as gel slice from the 0.7% agarose gel using a scalpel and placed in a preweighed microcentrifuge tube. Three volumes of the buffer QG (supplied by the manufacturer) were added and incubated at 50 $^{o}$C for 10 minutes or until the gel slice has completely dissolved. The gel mixture was vortexed every 2 to 3 minutes during the incubation period. The mixture should be in yellow after the gel is completely dissolved. The mixture was transferred into a QIAquick column inserted in a 2 ml collection tube and centrifuged at 12,000 rpm for 90 seconds. The flow-through solution was discarded. An another 500 µl of buffer QG was added to the QIAquick column and recentrifuged for 90 seconds. After this step, a 750 µl of buffer PE (supplied by the manufacturer) was added to the QIAquick column and centrifuged. The flow through solution was discarded. The QIAquick column was centrifuged to remove a trace amount of the washing solution. The QIAquick column was placed into a sterile 1.5 ml microfuge tube. DNA was eluted by an addition of 30 µl of buffer EB (10mM Tris-HCl. pH 8.5) or $H_2O$ to the center of the QIAquick membrane and let the column standing for 5 minutes, before centrifuged at 12,000 rpm for 90 seconds.

### 2.3.1.2 Preparation of vector DNA by digesting of pCAMBIA1301 with *Hin*dIII and *Eco*RI

Five micrograms of pCAMBIA1301 were digested with *Hin*dIII and *Eco*RI in the 100 µl reaction mixture using the condition as describe in section 2.3.1.1. The restricted product was then extracted once with phenol / chloroform / isoamyl alcohol (25:24:1) and once with chloroform. The DNA was precipitated with equal volume of absolute ethanol and dissolved in 20 µl of TE buffer.

### 2.3.1.3 Ligation of GFP fragment to pCAMBIA1301

The 1.5 kb DNA fragment of GFP gene was ligated to pCAMBIA1301 in 20 µl reaction containing 300 ng of DNA fragment, 100 ng of digested pCAMBIA1301, 1X T4 DNA ligase buffer (10 mM Tris-acetate, pH 7.5), 10 mM magnesium acetate and 50 mM potassium acetate), 1 mM of ATP, 5.5 weiss units of T4 DNA ligase (Pharmacia). The reaction mixture was incubated at 14 $^{o}$C overnight.

### 2.3.1.4 Transformation of ligated products to *E. coli* host cells by electroporation (Dower et al., 1988)

#### 2.3.1.4.1 Preparation of competent *E. coli* cells

A single colony of *E. coli* XL-1 BLUE was innoculated in 10 ml of LB-broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) and incubated with vigorous shaking at 37 $^{o}$C overnight. The starting culture was inoculated into 1 liter of L-broth and continued culture at 37 $^{o}$C with vigorous shaking to the $OD_{600}$ of 0.5 to 0.8. The cells were chilled briefly on ice for 15 to 30 minutes, and harvested by centrifugation in a prechilled rotor at 4,000 g for 15 minutes at 4 $^{o}$C. The pellet were resuspended in 1 liter of cold water and centrifuged as above. After resuspended in 0.5 liter of cold water, the pellet was centrifuged and resuspended in 20 ml of 10% glycerol. The cells were recentrifuged, and finally resuspended in 2 to 3 ml of 10% glycerol. This concentrated cell suspension was devided to 45 µl aliquots. These cells could be used immediately or stored at –80 $^{o}$C for later used.

#### 2.3.1.4.2 Electrotransformation of recombinant DNA to *E. coli* host cell

The competent cells were thawed on ice for 5 minutes. One or two microlitres of the ligation mixture was added and gently mixed by pipetting. The mixture was left on ice for approximately 1 minute. The mixture was eletroporated in a prechilled 0.2 cm cuvette using a Gene pulser (Bio-Rad) with the setting parameters of 25 µF 200Ω and 2.5 KV. After electroporation, the mixture were immediately removed

from the cuvette and added to a new tube containing 1 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) or LB broth. The cell suspension was incubated with shaking at 37 $^oC$ for 1 hours. Approximately 10-50 µl of this were spreaded on a selective LB agar plates containing 50 µg/ml of kanamycin, 25 µg/ml of IPTG and 20 µg/ml of X-Gal and further incubated at 37 $^oC$ overnight. The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

**2.3.1.4.3 Isolation of recombinant plasmid DNA**

Plasmid DNA was isolated using a modification of the alkaline lysis DNA method (Li et al., 1997). A white colony was inoculated into a sterile tube containing 5 ml of LB broth supplemented with 50 µg/ml of kanamycin and incubated with shaking at 37 $^oC$ overnight. The culture was transferred into a new 1.5 ml microcentrifuge tube and centrifuged at 10,000 rpm for 30 seconds. The supernatant was carefully decanted. One hundred microlitres of solution 1 (50 mM glucose, 10 mM EDTA, pH 8.0, 25 mM Tris-HCl, pH 8.0) was added to the cell pellet and vortexed. Two hundred microlitres of solution 2 (0.2 N NaOH and 1% SDS) was added and gently mixed by inversion of the tube. One hundred and fifty microlitres of solution 3 (3 M sodium acetate, pH 4.8) was added and mix by trapping of the tube. The tube was centrifuged at 10,000 rpm for 30 seconds to pellet cell debris. The supernatant was transferred into a new microcentrifuge tube. An equal volume of cold absolute ethanol was added and mixed by inversion following by centrifugation at 12,000 rpm for 10 minutes. The pellet was dried *in vacuo* for 5 minutes. The pellet was resuspended in 50 µl of TE buffer. RNaseA was added to a final concentration of 200 µg/ml to digest contaminating RNA. The reaction mixture was incubated at 37 $^oC$ for 30-60 minutes. Plasmid DNA was stored at –20 $^oC$.

**2.3.1.4.4 Detection of recombinant plasmid**

The existence of the insert DNA fragment was examined by digestion of recombinant DNA with *Hin*dIII and *Eco*RI. The reaction was carried out in a 20 μl standard mixture at 37 $^{o}$C overnight.

At the end of digestion period, the resulting product was electrophoretically analyzed by 1% agarose gel. The size of DNA insert is compare with that of a λ / *Hin*dIII and 200 bp DNA ladder. This recombinant plasmid is named pCAMBIA1306IC and will be used in rice transformation experiment.

**2.3.2 Transformation of pCAMBIA1301, pCAMBIA5305 and pCAMBIA1306IC to *A. tumefaciens* EHA105 host cells by electroporation**

**2.3.2.1 Preparation of competent *A. tumefaciens* EHA105 cells (Gelvin and Schilperoort, 1994)**

*A. tumefaciens* EHA105 was streaked on solid LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% NaCl and 1.5% Bacto agar) supplemented with 25 μg/ml rifampicin and incubated at 28 $^{o}$C for 2 days. A well-separated colony was innoculated in 10 ml of LB-broth supplemented with 25 μg/ml rifampicin and incubated with vigorous shaking at 28 $^{o}$C for 8 hours. The starting culture was inoculated into 1 liter of L-broth and continued culture at 28 $^{o}$C with vigorous shaking to the $OD_{600}$ of 1.0 to 1.5. The cells were harvested by centrifugation in a cold rotor at 4,000 g for 15 minutes. The pellet were resuspended in 1 liter of cold water and centrifuged as above. The supernatant was carefully poured off. After resuspended in 0.5 liter of cold water, the pellet was centrifuged and resuspended in 20 ml of 10% glycerol. The cells were recentrifuged, and finally resuspended in 1 ml of 10% glycerol. Usually, cells can be resuspended in the 10% glycerol that remains in the centrifuge bottle. This concentrated cell suspension was devided to 45 μl aliquots. These cells could be used immediately or can be frozen in a –80 $^{o}$C freezer for 6 months.

### 2.3.2.2 Electrotransformation of each binary vector to *A. tumefaciens* EHA105

The transformation vectors, pCAMBIA5305, pCAMBIA1301 and pCAMBIA1306IC were introduced into *A. tumefaciens* EHA105 by electrotransformation using condition as described above in section 2.3.1.4.2. The electroporated cells were immediately removed from the cuvette and added to a new tube containing of SOC medium. The cell suspension was incubated at 28 $^{o}$C with shaking for 2 hours. Approximately 10-50 µl of the cell suspension were spreaded on a selective LB agar plates containing 50 µg/ml of kanamycin, 25 µg/ml rifampicin and incubated at 28 $^{o}$C for 2 days.

### 2.3.2.3 Detection of binary vector

A white colony was inoculated into a sterile tube containing 5 ml of LB broth supplemented with 50 µg/ml of kanamycin and 25 µg/ml rifampicin with vigorous shaking at 28 $^{o}$C for 2 days. The plasmid DNA was extracted by a modification of alkaline lysis method as described in section 2.3.1.4.3. The pCAMBIA5305 and pCAMBIA1306IC were digested with *Hin*dIII and *Eco*RI to determine the size of insert GFP cassette using the condition as describe above in section 2.3.1.4.4. The pCAMBIA1301 was digested with *Bst*EII and *Eco*RI to determine the size of binary vector. These digested vectors were electrophoretically analyzed by 1% agarose gel.

## 2.4 Transformation of binary vectors to embryogenic calli by co-cultivation with *Agrobacterium*

### 2.4.1 Co-cultivation of embryogenic calli

*A. tumefaciens* strain EHA105 habouring pCAMBIA1301 and EHA105 containing pCAMBIA1306IC were streaked on solid AB medium (Appendix A) supplemented with 50 mg/ml hygromycin and 50 mg kanamycin whereas EHA105 habouring pCAMBIA5305 was streaked on solid AB medium supplemented with 50 mg/l kanamycin only. The bacteria were incubated at 28 $^{o}$C for 2 days and collected

by scraping from plates with platinum loop. The bacteria were resuspended in AAM medium (Appendix A) containing 100 μM of acetosyringone with vigorous shaking for a minute. The optical density of the bacterial suspension was adjusted to 0.01 by diluting with AAM medium. The embryogenic calli from section 2.2.1 were subcultured to fresh medium and incubated in the same condition for 4 days before using in co-cultivation. The four-day incubated embryogenic calli were immersed in bacterial suspension for 10 min with occasional shaking. The excess of bacteria was removed by decanting the liquid and calli were bloted dry on sterile filter papers (Whatman No. 1). The calli were then transferred to the co-cultivation medium (2NB-AS; 2NB supplemented with 100 μM of acetosyringone, Appendix A) and incubated in the dark at 25 $^{o}$C for three days.

## 2.5 Selection and regeneration of transformed calli

After the co-cultivation, the calli were removed from the co-cultivation medium and bloted dry on sterile filter papers whereas the *Agrobacterium* overgrowing calli were discarded. The co-cultivated calli were transferred to selection medium (2NB-CH; 2NB supplemented with 500 mg/l cefotaxime and 50 mg/l hygromycin; Appendix A) and incubated at 28 $^{o}$C for 4 weeks. The hygromycin resistant calli obtained after first round of selection were subcultured for two cycles onto fresh 2NB-CH medium every two weeks. The hygromycin resistant calli were then transferred to regeneration medium without any antibiotics (NB4-RE; NB-RE containing 4 mg/l 6-benzylaminopurine (BAP), Appendix A) and incubated at 28 $^{o}$C under 16 hours light photoperiod for 3-4 weeks. Green buds / shoots were observed after 4 week. When the transformed shoots become 2-3 cm in length, they were transferred to homone-free NB medium for stimulation of rooting and stem elongation for 4 weeks and transfer to soil for further growth.

## 2.6 Histochemical analysis for β -Glucuronidase (GUS) activity

GUS activity was assayed histochemically by a procedure based on that described by Jefferson, 1987 (Appendix B).

Histochemical GUS assays were made after co-cultivation of embryogenic calli with EHA105 (pCAMBIA1301) and also with putative transformed leaves and roots from EHA105 (pCAMBIA1306IC). The tissues were placed in microcentrifuge tube and 100 μl of histochemical (X-Gluc) staining solution was added. The tissues were vacuum infiltration for one minute and incubated at 37°C overnight. To facilitate detection of the blue color, 95% of ethanol was added to remove the chlorophyll.

## 2.7 Detection of GFP expression

### 2.7.1 Detection of GFP expression by fluorescent microscopy and a 365 nm hand-held ultraviolet lamp

Two system were used for GFP observation of transformed rice tissues. The first system consisted of fluorescent microscope and the second system consisted of a hand-held, long-wave ultraviolet lamp.

In the first system, visualization of GFP fluorescence in leaves, roots and hygromycin resistant calli was examined using an Olympus BX-50 fluorescent microscope fitted with a fluorescein isothiocyanate (FITC) filter set comprising excitation filter 450-490 nm, dichroic mirror 510 nm and barrier filter LP520 nm. The light source was provided by a HBO 50W high-pressure mercury bulb. Rice tissues were transferred to microscope slides and observed through 4X or 10X objectives. Photographs were taken using an Olympus automatic exposure photomicrographic system with Kodak Ektachrome 400 film. In the second system, hygromycin resistant calli were moved to the larminar flow hood and illuminated with a 365 nm hand-held ultraviolet lamp (Schleicher Schuell). Most lights were turned off. The photographs were taken using a 35-mm Kodak Ektachrome 400 film.

## 2.8 Analysis of GFP and *hpt* gene in total DNA of transformed rice

### 2.8.1 Rice DNA extraction using modified CTAB method (Weising et al., 1995)

Genomic DNA was extracted from control and putative transformed plants using the modified CTAB method.

The CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 0.2 M EDTA and 0.1 M Tris-HCl, pH 8.0) was preheated at 60 $^{o}$C. One gram of fresh plant tissue was harvested and grinded to a fine powder in liquid $N_2$ with mortar and pestle. The forzen powder was transferred to an organic solvent-resistant test tube. The 7.5 ml preheated CTAB extraction buffer was added to the tissue powder and mix thoroughly. The mixture was incubated at 65 $^{o}$C for 30 minutes with regularly swirling to ensure efficient extraction. The mixture was then extracted once with an equal volume of chloroform-isoamyl alcohol (25:24:1) by mixed gently for 15 minutes and centrifuged at 5,000 rpm for 10 minutes to separate phases. The upper aqueous phase was transferred to a new tube and 0.6 volume of ice-cold isopropanol was added and the mixture was incubated at –80 $^{o}$C for 10 minutes. The precipitated DNA pellet was recovered by centrifugation at 12,000 rpm for 15 minutes and briefly washed once with 70% ethanol. The pellet was air-dried and resuspended in 300 µl of TE buffer (10 mM Tris-HCl, pH7.4 and 1 mM EDTA). RNaseA was added to a final concentration of 200 µg/ml to digest contaminating RNA. The mixture was incubated at 37 $^{o}$C for 30 minutes. The DNA was then extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) by mixed gently for 15 minutes and centrifuged at 12,000 rpm for 10 minutes to separate phases. The upper aqueous phase was transferred to a new tube and further extracted twice with an equal volume of chloroform-isoamyl alcohol. One-tenth volume of 3 M sodium acetate pH 5.5 was added. DNA was precipitated by an addition of two volume of ice-cold absolute ethanol and incubated at –80 $^{o}$C for 15 minutes. The precipitated DNA pellet was recovered by centrifugation at 12,000 rpm for 15 minutes and briefly washed once with 70% ethanol. The pellet was air-dried and resuspended in 150 µl of TE buffer.

### 2.8.2 Measurement of DNA concentrations

The concentration of extracted DNA was spectrophotometrically measured at the optical density of 260 nanometre ($OD_{260}$). An $OD_{260}$ of 1.0 corresponds to a concentration of 50 μg/ml double stranded DNA. Therefore, the concentration of each samples (in μg/ml) was calculated by;

$$[DNA] = OD_{260} \text{ x dilution factor x } 50.$$

The purity of DNA samples can be obtained by calculating a ratio of $OD_{260}$ / $OD_{280}$. The ratio of 1.8 to 2.0 indicates pure prepared DNA whereas much higher and lower values of this ratio indicate RNA or protein contamination of isolated DNA samples, respectively (Sambrook, 1989).

### 2.8.3 Analysis of GFP fragment from putative transformants by polymerase chain reaction (PCR)

The presence of the GFP gene in putative transformed plants was assesed by PCR. Forward and reverse primers of GFP and *npt*II were designed using the Oligo 4.0 program. PCR conditions were optimized. The amplification reactions were performed in a 25 μl reaction volume containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 μM each of dATP, dCTP, dGTP and dTTP, 0.4 μM of each primer and 1.0 unit of Ampli *Taq* DNA polymerase. The reaction was performed in a DNA Thermal cycler (Perkin Elmer Model 2400) programmed for 30 cycles composing of a 94 $^{o}$C denaturation step for 1 minutes, a 68 $^{o}$C annealing step for 1 minutes and a 72 $^{o}$C extension step for 1 minutes. The final extension was carried out at 72 $^{o}$C for 5 minutes. Ten microliters of the amplification products are electrophoresed through 1.2% agarose gels and visualized by ethidium bromide staining.

# CHAPTER III
# RESULTS

## 3.1 Embryogenic callus induction

Callus is a mass of undifferentiated plant cells which, depending on the presence of different growth substances, can be induced to form shoots or roots (Walden, 1993). The callus induction media were compared between 2MS (MS medium supplemented with 2 mg/l 2,4-D; Appendix A) and 2NB (NB medium supplemented with 2 mg/l 2,4-D; Appendix A). Callus can initiate on both 2MS and 2NB media (Table 3.1). The calli were formed from the scutella tissue of mature seeds within 3-4 weeks of culture initiation. After 4 weeks, on 2MS induction medium, out of 43 of the average number of mature seed, 32 mature seeds produced calli corresponding to 73.9±0.5% calli induction. On 2NB medium, out of 54 of the average number of mature seed, 51 mature seeds produced calli corresponding to 93.9±4.8% calli induction.

Embryogenic callus, observed as being compact, yellowish, and granular, were composed of a majority of small meristematic cells with large nuclei, thin cell walls and high viability (Fig. 3.1). On 2MS induction medium, out of 43 of the average number of mature seed, 24 mature seeds produced embryogenic calli corresponding to 54.6±2.5% embryogenic calli induction. On 2NB medium, out of 54 of the average number of mature seed, 43 mature seeds produced embryogenic calli corresponding to 79.2±3.4% embryogenic calli induction. From this result, the embryogenic calli induction obtained from 2NB medium was higher than 2MS medium therefore, the 2NB medium was used for induction of embryogenic calli in subsequent experiments.

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**Figure 3.1** Embryogenic callus induction of *O. sativa* cv. KDML 105 after 4 weeks on 2NB medium

(A) Embryogenic callus

(B) Non-embryogenic callus (arrowhead)

**Table 3.1** Embryogenic callus induction efficiency from mature seeds of *Oryza sativa* cv. KDML 105 on 2MS and 2NB media

| Experiment | Media | Number of mature seed | | | | |
|---|---|---|---|---|---|---|
| | | Produced scutella (A) | Produced calli (B) | Produced embryogenic calli (C) | (%) Calli induction (B/A) | (%) Embryogenic calli induction (C/A) |
| 1 | 2MS | 45 | 32 | 24 | 71.1 | 53.3 |
| | 2NB | 54 | 47 | 42 | 87.0 | 77.8 |
| 2 | 2MS | 43 | 33 | 25 | 76.7 | 58.1 |
| | 2NB | 54 | 53 | 41 | 98.2 | 75.9 |
| 3 | 2MS | 42 | 31 | 22 | 73.8 | 52.4 |
| | 2NB | 55 | 53 | 46 | 96.4 | 83.7 |
| Average | 2MS | 43 | 32 | 24 | 73.9 ± (0.5) | 54.6 ± (2.5) |
| | 2NB | 54 | 51 | 43 | 93.9 ± (4.8) | 79.2 ± (3.4) |

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## 3.2 Regeneration of rice plant

The embryogenic calli were transferred onto regeneration media led to the formation of somatic embryos which gave rise to plantlets (Fig. 3.2). The MS1-RE (MS-RE medium supplemented with 1 mg/l BAP; Appendix A) and NB1-RE (NB-RE medium supplemented with 1 mg/l BAP; Appendix A) regeneration medium were compared. After 4 weeks of culture, green spots, shoots and roots from each regeneration medium were counted. The average number of embryogenic calli on medium MS1-RE was 64 calli, of these 19 calli produced green spots; corresponding to 29.5±2.1%; 18 calli produced shoots; corresponding to 27.4±2.9% and 38 calli produced roots; corresponding to 59.1±12.3%. On NB1-RE, the average number of embryogenic calli was 73 calli, of these 24 calli produced green spots; corresponding to 33.5±6.3%; 24 calli produced shoots; corresponding to 33.5±4.0% and 10 calli produced roots; corresponding to 13.3±4.6%. (Table 3.2). From this results, the NB1-RE medium gave the higher shoot formation than MS1-RE medium, therefore NB1-RE medium was used in subsequent experiment.

Four shoot regeneration media, with differing concentrations of BAP, were further tested in parallel for their ability to induce shoots on explants from embryogenic calli. Medium MS1-RE and NB1-RE contained 1 mg/l BAP whereas medium NB2-RE and NB4-RE had 2 and 4 mg/l BAP, respectively (Appendix A). After 4 weeks of culture, shoots from each regeneration medium were counted (Table 3.3). On MS1-RE, the average number of embryogenic calli was 20 calli, of these 5 calli produced green spots; corresponding to 22.5±7.5%; 6 calli produced shoots; corresponding to 27.5±3.8% and 15 calli produced roots; corresponding to 72.5± 7.5%. On NB1-RE, the average number of embryogenic calli was 21 calli, of these 7 calli produced green spots; corresponding to 34.5±5.7%; 7 calli produced shoots; corresponding to 31.7±1.5% and 3 calli produced roots; corresponding to 12.2±5.9%. On NB2-RE, the average number of embryogenic calli was 12 calli, of these 7 calli produced green spots; corresponding to 56.0±10.6%; 5 calli produced shoots; corresponding to 39.1±6.1% and 3 calli produced roots; corresponding to 21.7±3.4%. On NB4-RE, the average number of embryogenic calli was 11 calli, of these 4 calli produced green spots; corresponding to 36.4%; 8 calli produced shoots;

corresponding to 68.2±13.6% and 2 calli produced roots; corresponding to 13.6± 4.6%. Based on these results, media NB4-RE gave the highest shoot formation, therefore, NB4-RE was selected as the shoot regeneration medium used in the transformed experiment.

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**Figure 3.2** Regeneration of embryogenic callus of *O. sativa* cv. KDML 105 after 4 weeks on NB4-RE regeneration medium

(A) Embryogenic callus produced green spots (arrowhead)

(B1) Embryogenic callus produced roots (arrowhead)

(B2) Embryogenic callus produced shoots

**Table 3.2** Regeneration efficiency from embryogenic calli of *Oryza sativa* cv. KDML 105 on MS1-RE and NB1-RE media

| Experiment | Media | Number of embryogenic calli (A) | Produced green spots (B) | (%) Green spot formation (B/A) | Produced shoots (C) | (%) Shoot formation (C/A) | Produced roots (D) | (%) Root formation (D/A) |
|---|---|---|---|---|---|---|---|---|
| 1 | MS1-RE | 60 | 19 | 31.7 | 14 | 23.3 | 44 | 73.3 |
| | NB1-RE | 64 | 17 | 26.6 | 18 | 28.1 | 11 | 17.2 |
| 2 | MS1-RE | 73 | 22 | 30.1 | 22 | 30.1 | 32 | 43.8 |
| | NB1-RE | 82 | 26 | 31.7 | 31 | 37.8 | 6 | 7.3 |
| 3 | MS1-RE | 60 | 16 | 26.7 | 15 | 25.0 | 38 | 63.3 |
| | NB1-RE | 72 | 30 | 41.7 | 24 | 33.3 | 12 | 16.7 |
| Average | MS1-RE | 64 | 19 | 29.5± (2.1) | 18 | 27.4± (2.9) | 38 | 59.1± (12.3) |
| | NB1-RE | 73 | 24 | 33.5± (6.3) | 24 | 33.5± (4.0) | 10 | 13.3± (4.6) |

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**Table 3.3** Regeneration efficiency from embryogenic calli of *Oryza sativa* cv. KDML 105 on various media

| Experiment | Media | Number of embryogenic calli (A) | Produced green spots (B) | (%) Green spot formation (B/A) | Produced shoots (C) | (%) Shoot formation (C/A) | Produced roots (D) | (%) Root formation (D/A) |
|---|---|---|---|---|---|---|---|---|
| 1 | MS1-RE | 20 | 3 | 15.0 | 6 | 30.0 | 16 | 80.0 |
| | NB1-RE | 23 | 9 | 39.1 | 7 | 30.4 | 4 | 17.4 |
| | NB2-RE | 12 | 8 | 66.7 | 4 | 33.3 | 3 | 25.0 |
| | NB4-RE | 11 | 4 | 36.4 | 6 | 54.6 | 1 | 9.1 |
| 2 | MS1-RE | 20 | 6 | 30.0 | 5 | 25.0 | 13 | 65.0 |
| | NB1-RE | 18 | 5 | 27.8 | 6 | 33.3 | 1 | 5.6 |
| | NB2-RE | 11 | 5 | 45.5 | 5 | 45.5 | 2 | 18.2 |
| | NB4-RE | 11 | 4 | 36.4 | 9 | 81.8 | 2 | 18.2 |
| Average | MS1-RE | 20 | 5 | 22.5± (7.5) | 6 | 27.5± (3.8) | 15 | 72.5± (7.5) |
| | NB1-RE | 21 | 7 | 34.5± (5.7) | 7 | 31.7± (1.5) | 3 | 12.2± (5.9) |
| | NB2-RE | 12 | 7 | 56.0± (10.6) | 5 | 39.1± (6.1) | 3 | 21.7± (3.4) |
| | NB4-RE | 11 | 4 | 36.4± (0.0) | 8 | 68.2± (13.6) | 2 | 13.6± (4.6) |

## 3.3 Detection of GFP and GUS reporter gene combination

Approximately 5 µg of each extracted pCAMBIA1301 and pCAMBIA5305 was digested with both *Hin*dIII and *Eco*RI, resulting in linearized of pCAMBIA1301 (11.8 kb) and pCAMBIA5305 (10.4 kb) and GFP fragment (1.5 kb). The GFP fragment was separated by 0.7% agarose gel electrophoresis and eluted with QIAquick Gel Extraction Kit and ligated into *Hin*dIII/*Eco*RI site of pCAMBIA1301. The recombinant clones were extracted by alkaline extraction and then digested with *Hin*dIII and *Eco*RI to determine the corrected insert fragment. The insert fragment size of pCAMBIA1306IC was determined as 1.5 kb (Fig. 3.3). The structure and restriction maps of binary vectors pCAMBIA1301, pCAMBIA5305 and pCAMBIA1306IC are given in Appendix C.

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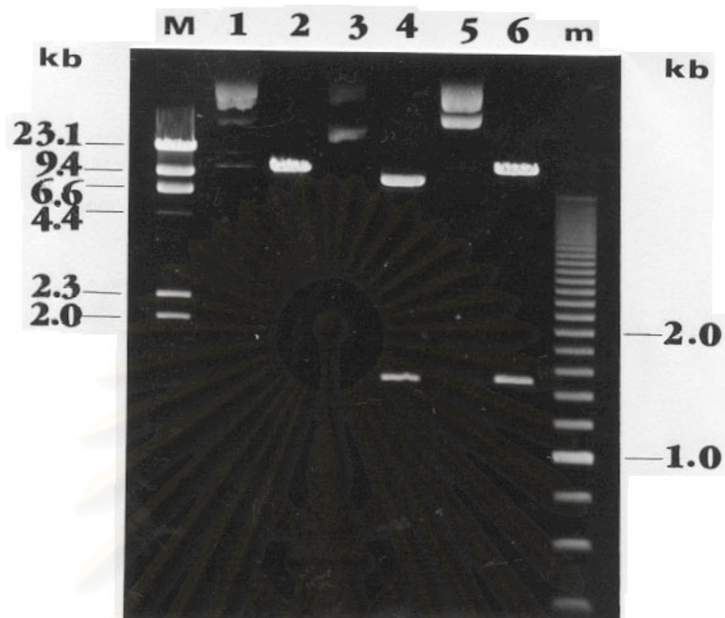


**Figure 3.3** Characterization of 1.5 kb inserted of GFP fragment in pCAMBIA1306IC on 1.0% agarose gel

Lane M = λ / *Hin*dIII standard marker

Lane 1 = undigested pCAMBIA1301 vector

Lane 2 = pCAMBIA1301 vector linearized with *Hin*dIII and *Eco*RI

Lane 3 = undigested pCAMBIA5305

Lane 4 = *Hin*dIII and *Eco*RI digested pCAMBIA5305

Lane 5 = undigested pCAMBIA1306IC

Lane 6 = *Hin*dIII and *Eco*RI digested pCAMBIA1306IC

Lane m = a 200 bp DNA ladder

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## 3.4 Co-cultivation of rice calli with *A. tumefaciens*

*A. tumefaciens* strain EHA105 habouring pCAMBIA1301, a GUS-expression construct, was used for co-cultivation of embryogenic calli. The embryogenic calli were co-cultivated for 3 days and GUS expression was examined immediately. The embryogenic calli showed histochemically GUS-positive blue spots (Fig. 3.4A). Out of 38 calli co-cultivated, six GUS positive calli were obtained. The transformation efficiency, defined as number of independent transformants divided by the number of explants inoculated, as a percentage, was 15.8% (Table 3.4). Control treatment never yielded GUS-positive blue spots.

The co-cultivated calli were transferred to the selection medium (2NB-CH) containing hygromycin (50 mg/l) to inhibit growth of non-transformed rice cells and supplemented with cefotaxime (500 mg/l) to inhibit *A. tumefaciens* growth. Continuous selection on hygromycin-containing medium resulted in the appearance of proliferating, apparently resistant, embryogenic calli. Hygromycin resistant calli were obtained after 4 weeks selection. These growing calli were excised and transferred to fresh selection medium and incubated in dark at 28 $^{o}$C for 4 weeks. The uninoculated control embryogenic calli did not show continuous growth, turned brown and died in selection medium (Fig.3.5A). After 8 weeks on selection with hygromycin (Fig. 3.5B), 38.7% of hygromycin resistant calli were obtained (Table 3.5). The segments of resistant calli were subjected to histochemical staining for GUS activity and many regions of uniformed intense blue were observed (Fig. 3.4 B and C). The frequency of transformed calli, based upon hygromycin-resistance and GUS activity, 19.4%, was observed from calli co-cultivated.

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**Table 3.4** Expression of GUS in rice callus immediately after co-cultivation with *A. tumefaciens* EHA105 (pCAMBIA1301)

| $OD_{600}$ | Duration of co-cultivation (days) | Number of pieces of tissue | | |
|---|---|---|---|---|
| | | Co-cultivated | Produced GUS+ | (%) GUS+ |
| 0.01 | 3 | 38 | 6 | 15.8 |

**Table 3.5** The recovery of GUS-expressing (GUS+), hygromycin-resistant (HygR) cells co-cultivation with *A. tumefaciens* EHA105 (pCAMBIA1301) on selective medium for 8 weeks

| $OD_{600}$ | Duration of co-cultivation (days) | Number of pieces of tissue | | | | |
|---|---|---|---|---|---|---|
| | | Co-cultivated; (A) | Produced HygR cells; (B) | Produced GUS+, HygR cells; (C) | (%) HygR; (B/A) | (%) GUS+, HygR; (C/A) |
| 0.01 | 3 | 191 | 74 | 37 | 38.7 | 19.4 |

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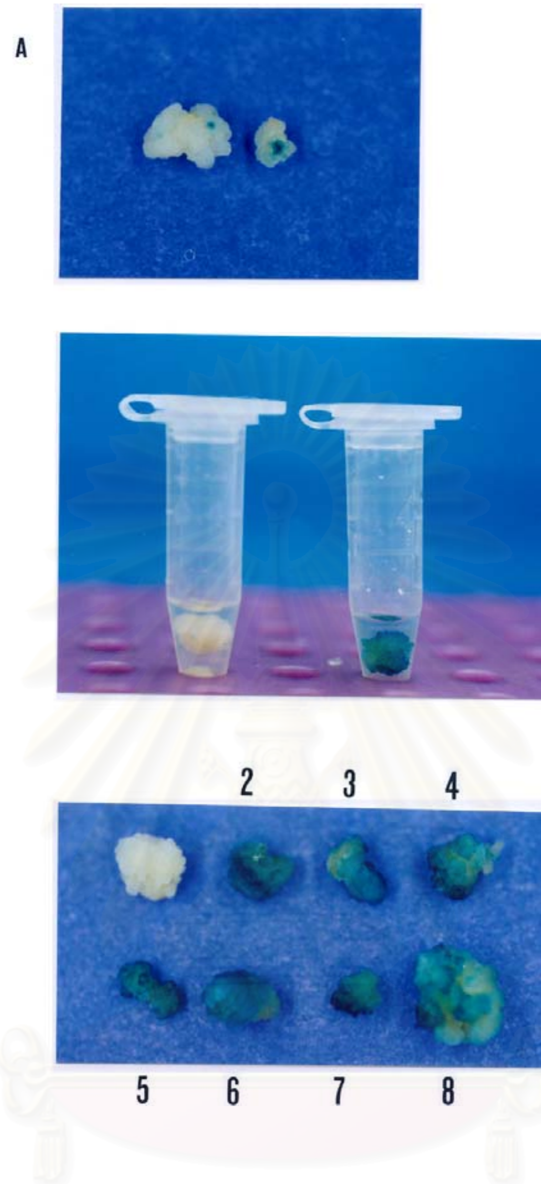


**Figure 3.4** Histochemical analysis of embryogenic callus transferred with *A. tumefaciens* EHA105 (pCAMBIA1301)

(A) Expression of GUS in embryogenic callus after co-cultivation for 3 days

(B) and (C) Uniform expression of GUS in hygromycin-resistant callus 8 weeks on selection medium

(C1) Control nontransformed callus

(C2-C8) Uniform expression of GUS in hygromycin-resistant callus 8 weeks on selection medium

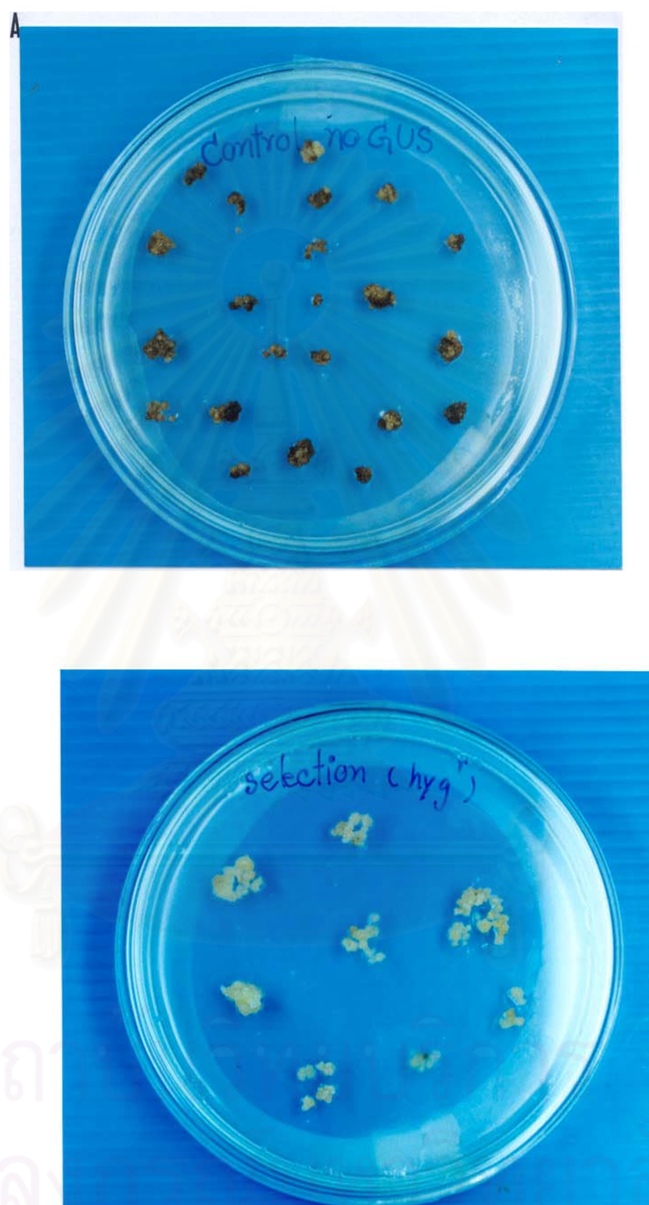


**Figure 3.5** Proliferation of the infected callus under hygromycin selection (50 mg/ml). Rice embryogenic callus transformation mediated by *A. tumefaciens* EHA105 (pCAMBIA1301)

(A) Brown zone of non-transformed embryogenic callus

(B) Resistant embryogenic callus growing on selective hygromycin medium after 8 weeks on selection medium

## 3.5 Production of GFP transformed calli

In order to recover the GFP transformed calli, the embryogenic calli were co-cultivated with *A. tumefaciens* EHA105 (pCAMBIA5305) or EHA105 (pCAMBIA1306IC). The components of plasmids used for rice transformation are shown in Appendix C. These plasmids have the *hpt*-intron, *hpt* and GFP gene in the T-DNA. The hygromycin resistant calli were obtained after 4 weeks selection. These calli were subcultured onto second round of selection on fresh selection medium for a further 4 weeks. Out of 92 calli co-cultivated, 30 hygromycin resistant calli were obtained corresponding to 32.6% transformation efficiency. After 8 weeks on selection medium (Fig. 3.6), analysis of transformed embryogenic calli was performed by fluorescent microscopy with multifilter (Fig. 3.7A) and FITC filter (Fig. 3.7B) using the same criterion (hygromycin resistance and GFP positive) for scoring plant transformation. Bright-green fluorescent calli were observed growing on hygromycin selection. No green fluorescence was observed in untransformed control calli. Out of 92 calli co-cultivated, 16 hygromycin resistant and GFP fluorescent calli were obtained corresponding to 17.4% transformation efficiency (Table 3.6). Moreover, when GFP transformed calli were illuminated with a hand-held, long-wave UV lamp, fluorescence was readily detectable by eye (Fig. 3.10A). In contrast, no green fluorescence was observed in non-transform control calli (Fig. 3.10B).

When the embryogenic calli were co-cultivated with *A. tumefaciens* EHA105 (pCAMBIA1306IC), having the *hpt*, GUS-intron and GFP gene in the T-DNA, the hygromycin resistant calli were brightly green fluorescent (Fig. 3.8). Out of 61 calli co-cultivated, 18 hygromycin resistant calli were obtained corresponding to 29.5% transformation efficiency, of these 10 calli were also GFP fluorescence and positive for GUS staining (Fig. 3.9) corresponding to 16.4% (Table 3.7).

**Table 3.6** Efficiency of rice transformation by *A. tumefaciens* EHA105 (pCAMBIA5305)

| $OD_{600}$ | Duration of co-cultivation (days) | Number of embryogenic calli | | | | | | | | Frequency (E/A, %) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Co-cultivated (A) | Produced HygR cells (B) | Produced HygR / GFP+ (C) | (%) HygR, (B/A) | (%) HygR, GFP+ (C/A) | Produced plants (D) | (%) Regeneration (D/C) | Produced GFP+ plants (E) | |
| 0.01 | 3 | 92 | 30 | 16 | 32.6 | 17.4 | 9 | 56.3 | 9 | 9.8 |

**Table 3.7** Efficiency of rice transformation by *A. tumefaciens* EHA105 (pCAMBIA1306IC)

| $OD_{600}$ | Duration of co-cultivation (days) | Number of embryogenic calli | | | | | | | | Frequency (G/A, %) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Co-cultivated (A) | Produced HygR cells (B) | Produced HygR/ GFP+/ GUS+ (C) | (%) HygR, (B/A) | (%) HygR/ GFP+ /GUS+ (C/A) | Produced plants (E) | (%) Regeneration (E/C) | Produced GFP+ / GUS+plants (G) | |
| 0.01 | 3 | 61 | 18 | 10 | 29.5 | 16.4 | 5 | 50 | 5 | 8.2 |

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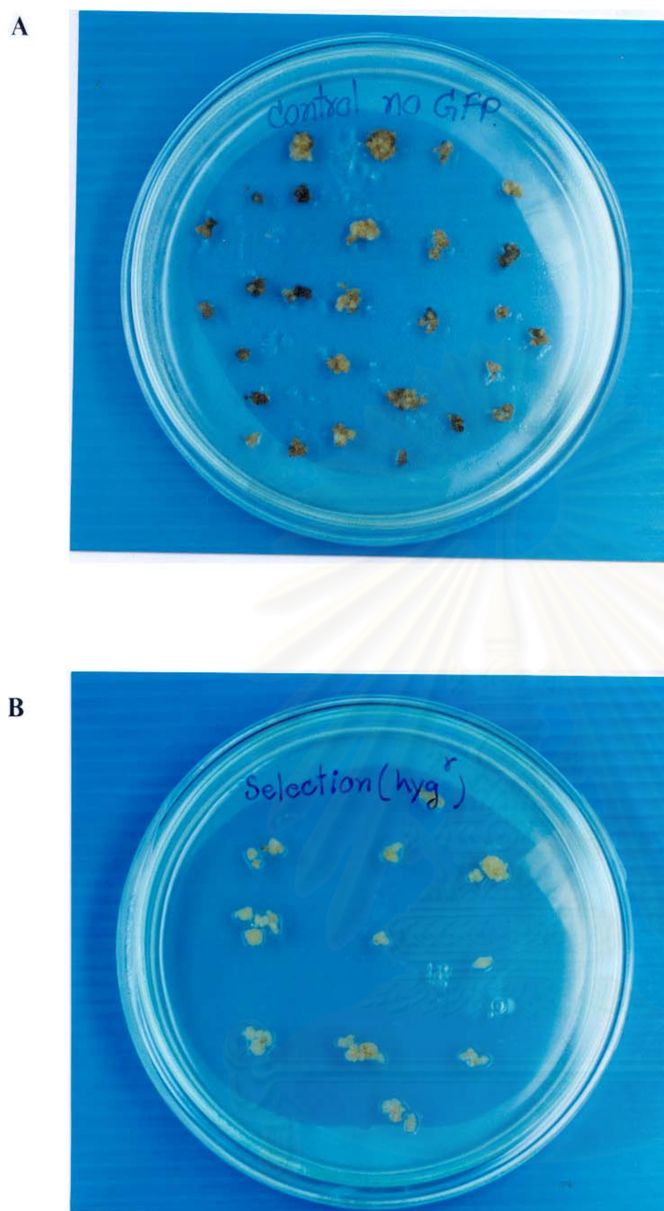


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**Figure 3.6** Proliferation of the infected callus under hygromycin selection (50 mg/ml). Rice embryogenic callus transformation mediated by *A. tumefaciens* EHA105 (pCAMBIA5305)

(A) Brown zone of non-transformed embryogenic callus

(B) Resistant embryogenic callus growing on selective hygromycin medium after 8 weeks on selection medium

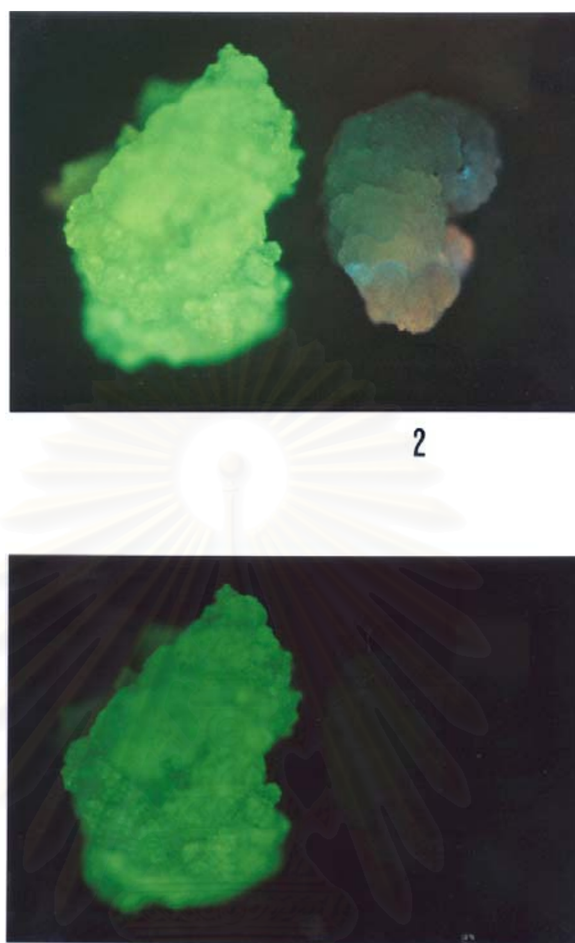


**Figure 3.7** GFP as a vital screenable marker in rice transformation using EHA105 (pCAMBIA5305). Observations were performed using a fluorescent microscope.

(A1) Green fluorescence of transformed embryogenic callus 8 weeks on selection medium observed under the fluorescent microscope using multifilter

(A2) Autofluorescence of non-transformed embryogenic callus 8 weeks on selection medium observed under the fluorescent microscope using multifilter

(B1) The same green fluorescence of transformed embryogenic callus 8 weeks on selection medium observed under the fluorescent microscope using fluorescein isothiocyanate (FITC) filter

(B2) The same non-transformed embryogenic callus 8 weeks on selection medium observed under the fluorescent microscope using fluorescein isothiocyanate (FITC) filter

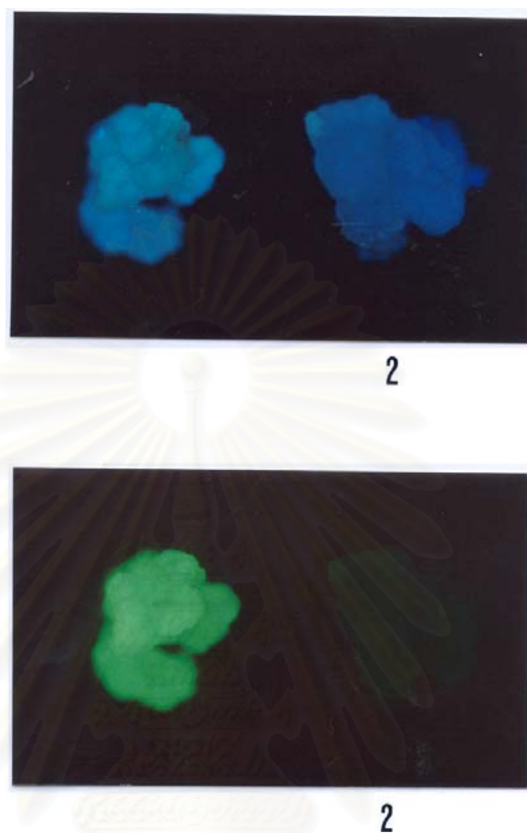


**Figure 3.8** GFP as a vital screenable marker in rice transformation using EHA105 (pCAMBIA1306IC). Observations were performed using a fluorescent microscope.
(A1) Transformed embryogenic callus 8 weeks on selection medium observed under the fluorescent microscope using blue filter
(A2) Non-transformed embryogenic callus 8 weeks on selection medium observed under the fluorescent microscope using blue filter
(B1) The same transformed callus show green fluorescence observed under the fluorescent microscope using fluorescein isothiocyanate (FITC) filter
(B2) The same non-transformed embryogenic callus observed under the fluorescent microscope using fluorescein isothiocyanate (FITC) filter

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**Figure 3.9** Histochemical analysis of hygromycin resistant callus transferred with *A. tumefaciens* EHA105 (pCAMBIA1306IC) after 8 weeks on selection medium

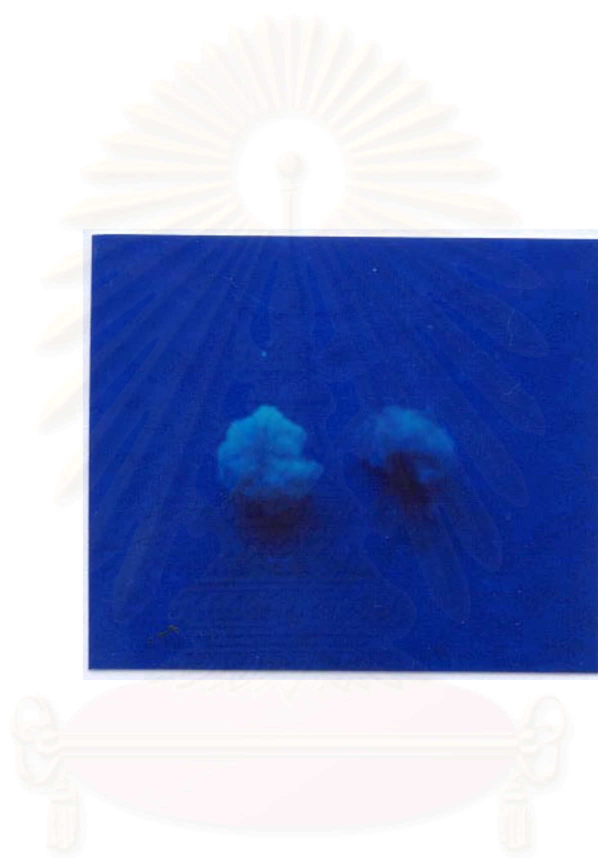

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**Figure 3.10** Detection of GFP in transformed callus illuminated with a hand-held, long-wave UV lamp

(A) Transformed callus

(B) Non-transformed callus

## 3.6 Regeneration of putative GFP transformed calli

Most of the colonies of cells that recovered from the first round of selection proliferated on the second round selective medium. Transgenic rice plants were regenerated from visually selected GFP-positive calli. Plants were readily regenerated when the resistant cells were transferred to a regeneration medium. Although stringent selection was maintained through callus growth, the hygromycin was not included during regeneration. The formation of green buds/shoots from somatic embryos occurred within 3 weeks of transferring hygromycin resistant calli onto NB4-RE regeneration medium. Healthy plantlets with extensive root systems were established on NB medium without any plant hormone after another 2-3 weeks (Fig. 3.11A). The frequency of regeneration ranged from 50 to 56% of selected colonies (Table 3.6 and Table 3.7). No differences were observed in the morphology between transformed and untransformed plants. These putative transgenics were subsequently transferred to soil (Fig. 3.11B). The transgenic nature of these plants were confirmed by histochemical localization of GUS gene expression, GFP fluorescence and PCR analysis. A total of 14 plants could be regenerated from GFP-positive and hygromycin-resistant calli of KDML 105. Of these, 9 were from calli transformed with EHA105 (pCAMBIA5305) and 5 were from calli transformed with EHA105 (pCAMBIA1306IC). Regenerated plants were also tested for GFP fluorescence. GUS activity was tested for regenerated plant from calli transformed with EHA105 (pCAMBIA1306IC).

## 3.7 Expression of GFP in primary transformed rice plants

Transgenic rice plants were regenerated from visually selected GFP-positive calli. The embryoid shoot primordia and the emerging primary shoots showed uniformed GFP fluorescence (Fig. 3.12). As development progressed, GFP expression was limited in chlorophytic tissues, such as developed leaves (Fig. 3.13). GFP expression was more readily identified in emerging roots since these tissues lack chlorophyll (Fig. 3.14 and 3.15). No GFP fluorescence was detected in the corresponding parts of non-transformed plants. No differences were observed in the morphology between transformed and untransformed plants. Of the 9 transformants

transformed with EHA105 (pCAMBIA5305) and 5 transformants transformed with EHA105 (pCAMBIA1306IC); all of these showed GFP expression. The transgenic nature of these plants were confirmed by PCR of GFP gene.

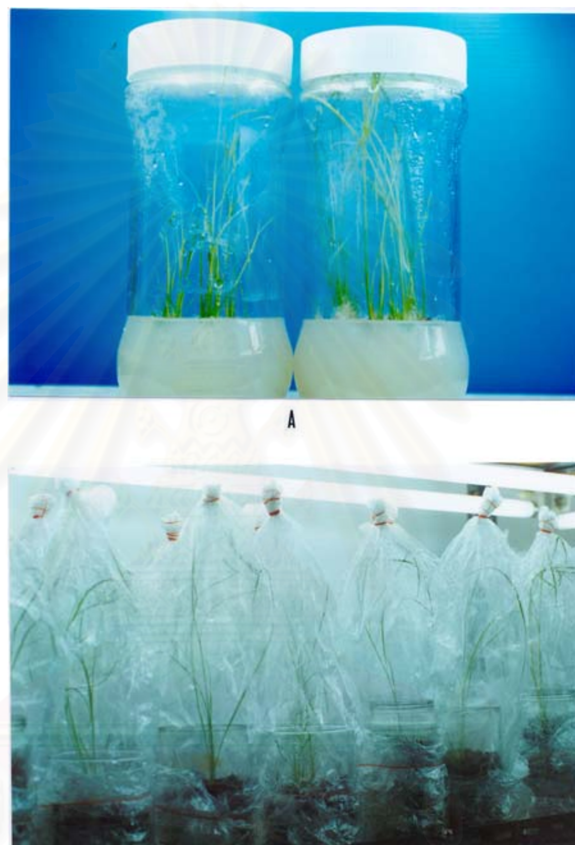


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**Figure 3.11** Regeneration of  transformants

(A) Regenerated transgenic rice plants after transfer to NB medium (rooting medium) for 4 week

(B) Regenerated transgenic rice plants with extensive root system transferred to soil for further growth

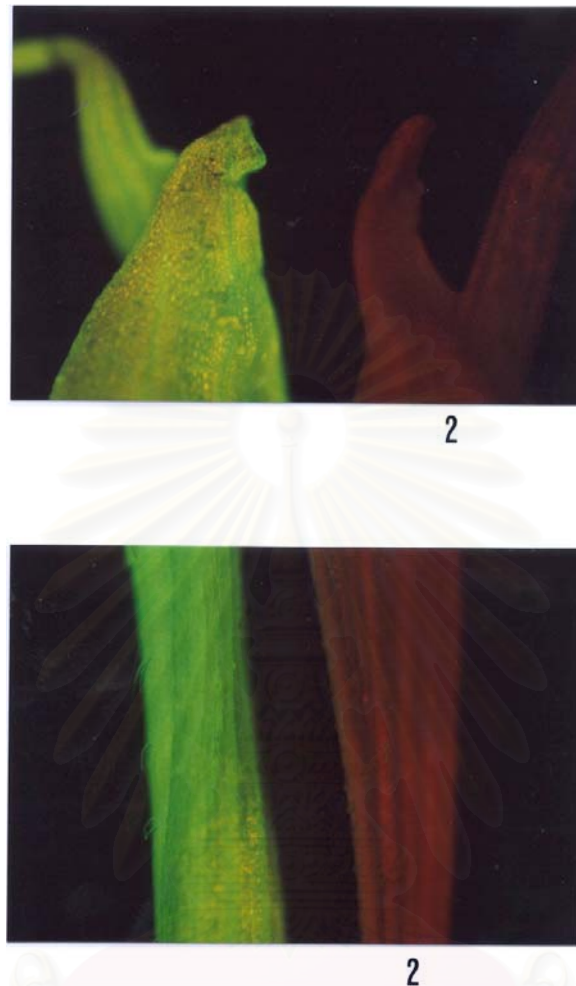


**Figure 3.12** GFP expression in primary transformants

(A1) GFP-expressing shoot primordia 4 weeks after regeneration on NB4-RE regeneration medium observed under fluorescent microscope using FITC filter

(A2) Autofluorescence of non-transformed shoot primordia 4 weeks after regeneration on NB4-RE regeneration medium observed under fluorescent microscope using FITC filter

(B1) GFP-expressing leaves 4 weeks after regeneration on NB4-RE regeneration medium observed under fluorescent microscope using FITC filter

(B2) Autofluorescence of non-transformed leaves 4 weeks after regeneration on NB4-RE regeneration medium observed under fluorescent microscope using FITC filter

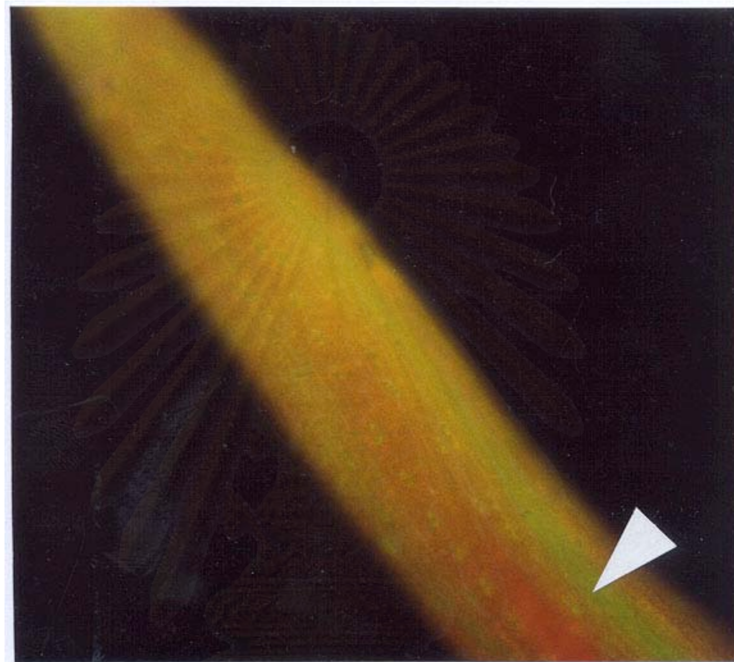

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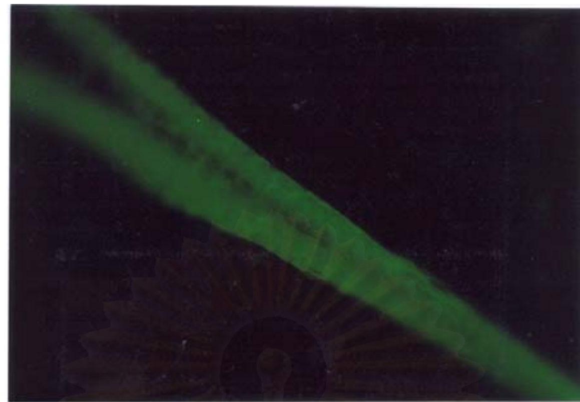
**Figure 3.13** Detection of GFP in the leaves of transgenic rice plants 12 weeks after regeneration



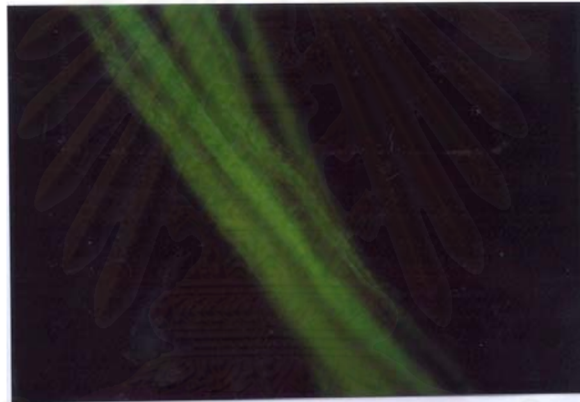


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**Figure 3.14** (A) and (B) Detection of GFP in roots from different samples of transgenic rice plants 12 weeks after regeneration

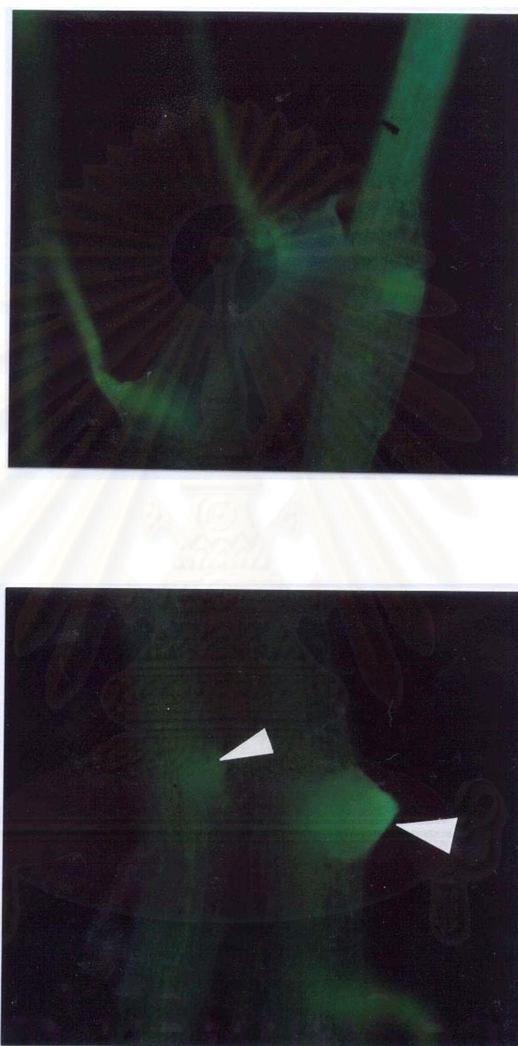

**Figure 3.15** (A) and (B) Expression of GFP in root and root nodules (arrowhead) from different samples of transgenic rice plants 12 weeks after regeneration

## 3.8 Histochemical analysis of GUS expression in primary transformants

GUS expression was detected in putative transformants obtained using EHA105 (pCAMBIA1306IC). The histochemical assay for GUS activity in different organs of primary transformants revealed intense blue staining in roots (Fig. 3.16B) and at the cut surfaces of the leaf pieces (Fig. 3.16A) which probably because of the lack of tissue penetrability by the X-Gluc substrate. Tissues from control plants did not show GUS expression (Fig. 3.16). Of the 5 plants regenerated, all tested positive for GUS.

## 3.9 DNA extraction

The total DNA of rice was extracted using a modified CTAB extraction method. The DNA was shown as a high molecular weight DNA (> 23.1 kb) (Fig. 3.17). DNA concentrations were spectrophotometrically determined by measuring the optical density at 260 nm. An $OD_{260}$ of 1.0 corresponds to a concentration of 50 μg DNA/ml double stranded DNA. Approximately 50 μg - 100 μg of nucleic acids were obtained from 1 g starting rice plant tissue. The ratio of $OD_{260}$ / $OD_{280}$ was 1.8 – 2.0 indicates pure prepared DNA.

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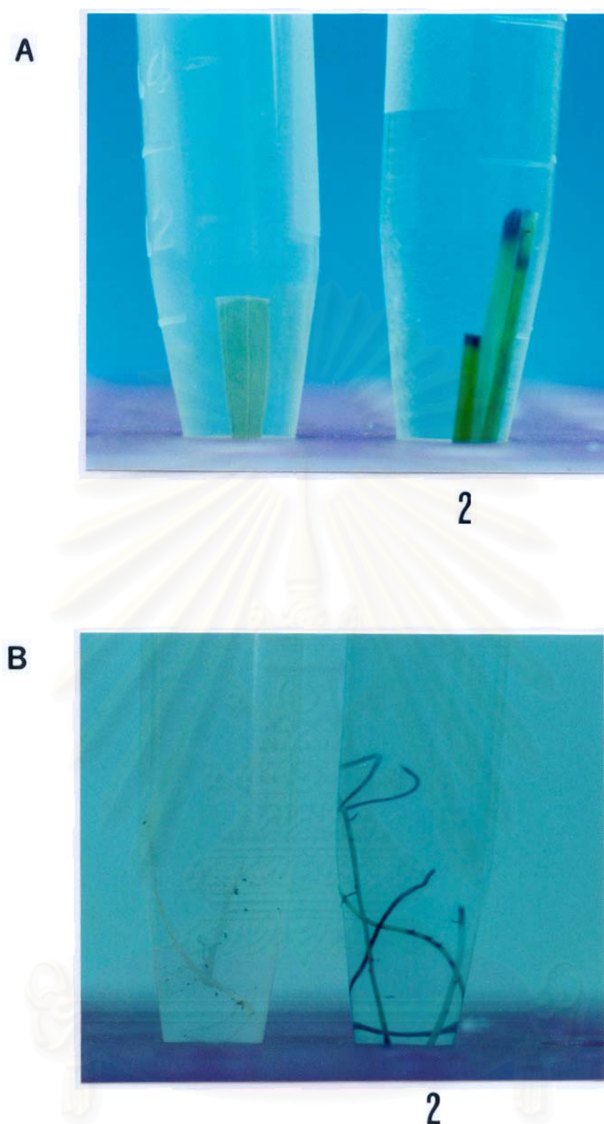


**Figure 3.16** Histochemical analysis of primary transformants co-cultivated with *A. tumefaciens* EHA105 (pCAMBIA1306IC)

(A1) No expression of GUS from leaves segment of the non-transformed plant

(A2) Expression of GUS showed blue staining at cut surface from leaves of the transformed plant

(B1) No expression of GUS from roots segment of the non-transformed plant

(B2) Expression of GUS showed uniform blue staining in the roots segment of the transformed plant

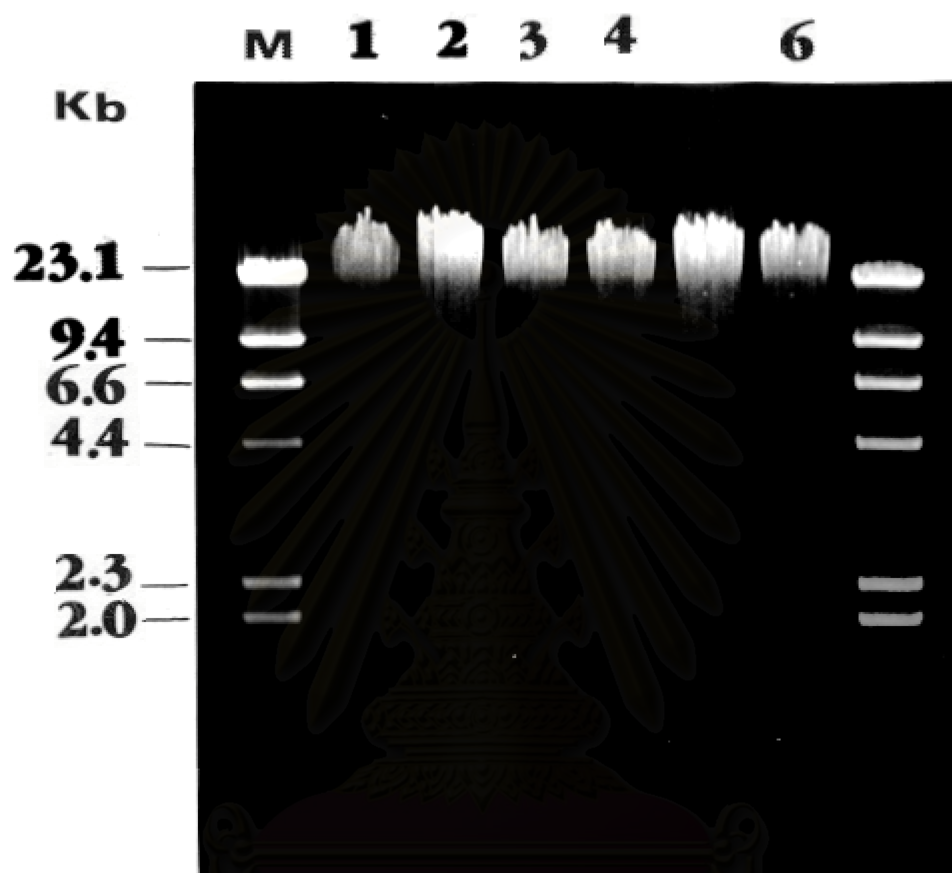


**Figure 3.17** A 1.0 % ethidium bromide stained - agarose gel showing the quality of total DNA extracted from rice plant tissues

lane M = λ / *Hin*dIII

lanes 1-6 = Total DNA extracted of rice plants

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## 3.10 PCR analyses of putative transformants

Molecular analysis by PCR amplification confirmed that the GFP gene was present in the putative transformed rice plants. GFP sequence is shown in Fig. 3.18. A pair of primers from sGFP and *npt*II gene were designed (Table 3.8). Genomic DNA from KDML 105 putative transformants resulting from infections with EHA105 (pCAMBIA5305) and EHA105 (pCAMBIA1306IC) was amplified with GFP gene primers as well as *npt*II gene primers (Fig. 3.19-Fig. 3.20). One bands corresponding to the expected GFP fragments of 720 bp was detected in all the putative transformants tested. No band was detected in case of negative control DNA; DNA of uninfected rice tissues. Since *npt*II gene is present outside the T-DNA in pCAMBIA5305 and pCAMBIA1306IC, *npt*II gene primers were used to check the band of 707 bp for the presence of any contaminating *Agrobacterium* cells in the plant tissue, although amplication could also result from T-DNA transfer that extends beyond the T-DNA border. However, none of the plants analysed showed amplification of the *npt*II gene sequences eliminating the possibility of bacterial contamination in the transformed tissues. The transformation efficiency determined by transgenic plants that stably expressed GFP fluorescence or GUS activity relative to the number of pieces of scutellum-derived calli that had been co-cultivated with bacterial cells was 8.2 and 9.8% (Table 3.6 and Table 3.7).

**Table 3.8** Sequences of oligonucleotide primers designed from sGFP and *npt*II genes

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| | **Primer sequence** |
|---|---|
| **sGFP gene** | Forward primer : 5′- **ATG GTG AGC AAG GGC GAG GAG C** -3′<br>Reverse primer : 5′- **TTA CTT GTA CAG CTC GTC CAT GCC**-3′ |
| ***npt*II gene** | Forward primer : 5′- **AAA ACT GAT CGA AAA ATA CCG CTG C**-3′<br>Reverse primer : 5′- **TCC CCA GTA AGT CAA AAA ATA GCT C**-3′ |

```
   Forward-sGFP primer
atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg gtc gag ctg gac 60
M   V   S   K   G   E   E   L   F   T   G   V   V   P   I   L   V   E   L   D

ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc gag ggc gag ggc gat gcc acc tac 120
G   D   V   N   G   H   K   F   S   V   S   G   E   G   E   G   D   A   T   Y

ggc aag ctg acc ctg aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc 180
G   K   L   T   L   K   F   I   C   T   T   G   K   L   P   V   P   W   P   T

ctc gtg acc acc ttc acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag 240
L   V   T   T   F   T   Y   G   V   Q   C   F   S   R   Y   P   D   H   M   K

cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag cgc acc atc ttc 300
Q   H   D   F   F   K   S   A   M   P   E   G   Y   V   Q   E   R   T   I   F

ttc aag gac gac ggc aac tac aag acc cgc gcc gag gtg aag ttc gag ggc gac acc ctg 360
F   K   D   D   G   N   Y   K   T   R   A   E   V   K   F   E   G   D   T   L

gtg aac cgc atc gag ctg aag ggc atc gac ttc aag gag gac ggc aac atc ctg ggg cac 420
V   N   R   I   E   L   K   G   I   D   F   K   E   D   G   N   I   L   G   H

aag ctg gag tac aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac 480
K   L   E   Y   N   Y   N   S   H   N   V   Y   I   M   A   D   K   Q   K   N

ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc gtg cag ctc gcc 540
G   I   K   V   N   F   K   I   R   H   N   I   E   D   G   S   V   Q   L   A

gac cac tac cag cag aac acc ccc atc ggc gac ggc ccc gtg ctg ctg ccc gac aac cac 600
D   H   Y   Q   Q   N   T   P   I   G   D   G   P   V   L   L   P   D   N   H

tac ctg agc acc cag tcc gcc ctg agc aaa gac ccc aac gag aag cgc gat cac atg gtc 660
Y   L   S   T   Q   S   A   L   S   K   D   P   N   E   K   R   D   H   M   V

ctg ctg gag ttc gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag taa 720
L   L   E   F   V   T   A   A   G   I   T   L   G   M   D   E   L   Y   K   *
                                                         Reverse sGFP-primer
```

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**Fig. 3.18** Nucleotide and amino acid sequences of sGFP gene. The locations and sequences of sGFP-specific forward and complementrary reverse primers are labelled in bold face and underlined

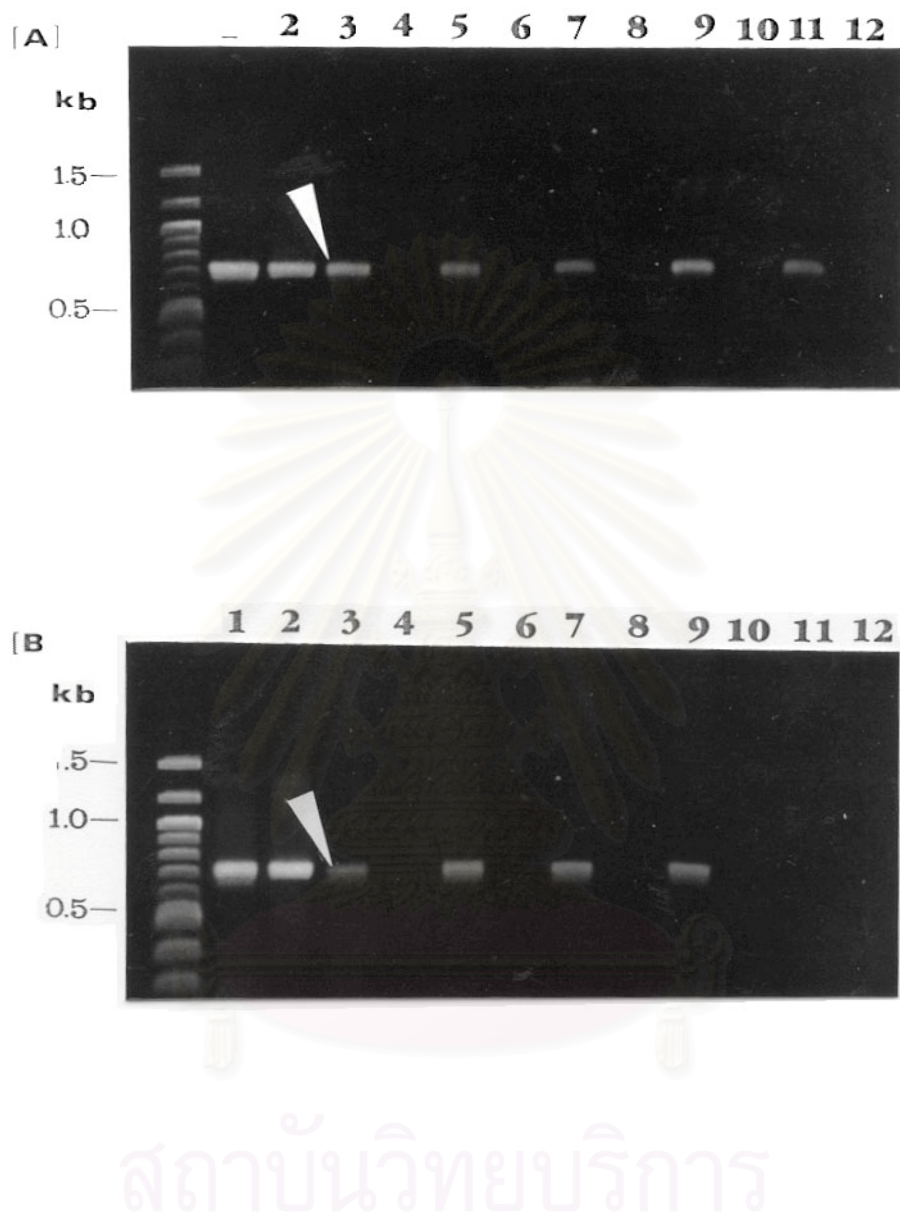


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**Figure 3.19** PCR analysis of the GFP gene in putative transformed rice plants co-cultivated with *A. tumefaciens* EHA105 (pCAMBIA5305) at $OD_{600}$ = 0.01 for 3 days. Total DNA isolated from whole plants was subjected to PCR amplification with GFP specific primers (lane 3, 5, 7, 9, 11; A and lane 3, 5, 7, 9; B) showing the presence of an expected 720 bp DNA fragment of GFP gene. Kanamycin specific primers were also used to check the band of 707 bp for the presence of any contaminating *Agrobacterium* cells in plant tissues (lane 4, 6, 8, 10, 12; A and lane 4, 6, 8, 10; B). The GFP gene and the kanamycin gene (positive control) were also amplified from the pCAMBIA5305 vector (lane 1 A, B and 2 A, B; respectively). DNA isolated from non-transformed plants was used as negative control for GFP and kanamycin specific primer (lane 11 and 12; B).

**Figure 3.20** PCR analysis of the GFP gene in putative transformed rice plants co-cultivated with *A. tumefaciens* EHA105 (pCAMBIA1306IC) at $OD_{600}$ = 0.01 for 3 days. Total DNA isolated from whole plants was subjected to PCR amplification with GFP specific primers (lane 3, 5, 7, 9, 11) showing the presence of an expected 720 bp DNA fragment of GFP gene. Kanamycin specific primers were also used to check the band of 707 bp for the presence of any contaminating *Agrobacterium* cells in plant tissues (lane 4, 6, 8, 10, 12). The GFP gene and the kanamycin gene (positive control) were also amplified from the pCAMBIA5305 vector (lane 1 and 2; respectively). DNA isolated from non-transformed plants was used as negative control for GFP and kanamycin specific primer (lane 13 and 14).

# CHAPTER IV
# DISCUSSION

Rice (*Oryza sativa* L.) is the major nutrition source for about 40% of the world's population, including many of the people living in the developing countries. Conventional plant breeding methods have contributed to great improvements to both *indica* and *japonica* rice varieties; however, genetic engineering provides an opportunity for introducing foreign genes that are not readily incorporated into new varieties by conventional breeding methods.

The introduction of foreign genes into monocots has so far depended on direct DNA transfer into protoplasts or cells. However, there are certain problem in the procedure for protoplast-mediated transformation of rice. It still remains difficult to regenerate plants from protoplasts of *japonica* and the majority of *indica* varieties (Abdullah et al., 1989). Moreover, plant regenerated from protoplasts are often phenotypically abnormal (Datta et al., 1992; Li et al., 1990).

In addition, direct DNA transfer into intact cells or tissues via particle bombardment was also reported. However, transgenic plants generated via this system frequently have multiple copies of transgenes integrated in the genome. This situation is often associated with unpredictable gene silencing and sterility (Finnegan and McElroy, 1994; Flavell, 1994).

*Agrobacterium*-mediated transformation offers some advantages in comparison with direct transformation methods such as the low copy integration, relatively precise mode of the DNA transfer, the high efficiency of transformation, the transfer of relatively large piece of DNA, the absence of a requirement for protoplast-culture techniques, the less expensive nature and being a simple procedure (Hiei et al., 1994). Systems of *Agrobacterium*-mediated transformation have been well established for many dicotyledonous plants. However, monocotyledonous plants, in particular cereal plants, were originally outside the host range of *A. tumefaciens.*

The apparent difficulties encountered in transforming cereals with *Agrobacterium* led Potrykus (1990) to conclude that this approach had very little potential for cereals. This study and the studies on rice, maize, wheat and barley transformation mediated by *A. tumefaciens* (Hiei et., 1994; Ishida et al., 1996; Cheng et al., 1997 and Tingay et al., 1997) have provided strong support that monocotyledons can be transformed as dicotyledons using *A. tumefaciens* by manipulating various factors such as appropriate starting material which is capable of active cell division leading to efficient plant regeneration, the addition of acetosyringone to the media, and the conditions of tissue culture and co-cultivation, as well as the *A. tumefaciens* strain.

The choice of a suitable explant source as starting material for infection of *Agrobacterium* is one of the most important factors. Hiei et al. (1994) compared various rice tissues, namely, shoot apices and segments of roots from young seedlings, scutella, immature embryos, calli induced from young roots and scutella and cells in suspension used for *Agrobacterium*-mediated transformation. Early expression of GUS was detected in all of the tissues examined apart from the root segments. The calli derived from scutella gave the highest ratio of GUS-expressing tissue to inoculated tissue. GUS expression was also observed in tissues that included shoot apices and immature embryos, tissues that were successfully transformed in previous studies (Chan et al., 1993 and Raineri et al., 1990). However, only a few transformants were obtained from immature embryos and none were obtained form shoot apices. The results of this study clearly indicate that callus cultures initiated from scutella are excellent materials for transformation of rice by *Agrobacterium*.

In this study, the calli initiated from scutella of mature seeds were used as starting material for *Agrobacterum*-mediated transformation of rice. Some reports used calli initiated from immature embryo (Aldemita and Hodges, 1996 and Dong et al., 1996) due to their better regeneration potential. However, the supply of immature embryos is environment-dependent (immature embryo was obtained after pollination 10-12 days) therefore, it is difficult to maintain a continuous supply of suitable explants (Li et al., 1993).

Callus formation is dependent on the presence in the culture medium of auxins and cytokinins. Initiation of embryogenic calli from mature seed of KDML 105 was obtained on both 2NB and 2MS media supplemented with 2 mg/l 2,4-dinitrophenoxyacetic acid (2,4-D) (artificial auxin). However, on 2NB medium, the percentage of embryogenic calli induction was higher than 2MS medium. In this study, the number of mature seed produced embryogenic callus was 79.2±3.4%. Both NB and MS medium composed of similar macronutrients and micronutrients which essential for plant growth but concentration of these nutrients were differ. Moreover, NB medium composed of L-proline which Kuleung (1997) reported the use of 10 mM of L-proline can promote embryogenic callus formation in KDML 105.

In addition, the use of young embryogenic calli as target explants could eliminate long tedious culture procedures and further problem with somaclonal variation (Larkin and Scowcroft, 1981). Moreover, embryogenic calli compose of actively dividing cells competent for *Agrobacterium* infection and are capable of regeneration into a whole plant. Precultured of embryogenic callus for 4 days on 2 mg/l 2,4-D medium prior to co-cultivation has been found to enhance the rate of cell division (An, 1985) and, thereby, making the tissue more competent for *Agrobacterium* infection (Vijaychandra et al., 1995).

The regeneration system via somatic embryogenesis has contributed significantly to the efficiency of the transformation procedure. Recently, Lee et al. (1999) reported the increasing of rice plant regeneration efficiency by water stress treatment. However, there was variability among cultivars; Dongjinbyeo, IR43, Nagdongbyeo and Sinseonchalbyeo showed a considerable increase in frequency of regenerable calli (18.3%-31.6%, 0.4%-48.7%, 44.1%-79.5% and 1.1%-32.6%, respectively), whereas Nonganbyeo and Sangjubyeo cultivars responded poorly to shoot regeneration even after water stress (0%-0.2% and 6.5%-11.2%).

In this result, two-month-old scutellum-derived calli of KDML 105 was also stressed by dehydration during plant regeneration by addition of 16 g/l Bacto agar to all of the regeneration medium instead of 8 g/l used in callus induction medium. In this study embryogenic calli from KDML 105 were regenerated on NB-RE medium

supplemented with 1, 2 and 4 mg/l 6-benzylaminopurine (BAP). BAP has been widely used as a cytokinin for stimulating shoot proliferaion in plant cell cultures. If the ratio of auxin to cytokinin was high in the growth media callus would be induced to form roots, whereas when the ratio of auxin to cytokinin was low shoots were formed. Intermediate ratios of auxins to cytokinins favoured maintained callus growth (Walden, 1993). BAP is generally used in plant cell culture at a concentration between 0.1-10 mg/l. When added in appropriate concentrations it may regulate cell division, stimulate axillary and adventious shoot proliferation, regulate differentiation, inhibit root formation, activate RNA synthesis and stimulate protein and enzyme activity (Li et al. 1993). From this result, NB4-RE regeneration medium containing 4 mg/l BAP gave the highest regeneration frequency approximate 60%.

Though KDML 105 shows as much as 60% regeneration of green plants from primary calli of scutella origin, the regeneration efficiency of transformed calli was lower than those non-transformed treatment. This was similar to results from studies of transformation of KL and IR-64 cultivar (Khanna and Raina, 1999). Khanna and Raina (1997) reported the regeneration efficiency of non-transformed KL and IR-64 show as much as 100% but the calli undergo a loss in regeneration potential resulted in approximately 15% (8/52) of transformed calli. The loss in regeneration potential in transformation procedure should have been resulted from the long term cultures and the length of time in culture in the presence of hygromycin, impaired plant regeneration (Raineri et al., 1990 and Peng et al., 1992). Moreover, *indica* cultivars are known to be recalcitrant to *in vitro* manipulations, largely due to a rapid decline in regenerability with increasing age in culture.

Tinjuangjun (1996) reported the transformation of Thai rice varieties with gene relating to diesease resistance using particle bombardment method. Calli and scutella of KDML 105 were bombarded with pIG121Hm and pUBA plasmids. Although resistant calli were obtained, the regeneration of transformed calli was not yet successful.

Monocotyledons, in particular grasses, appear not to produce phenolic compounds, or if they do, the levels are insufficient to serve as signals (Smith and

Hood, 1995). For this reason, they have been considered recalcitrant to *A. tumefaciens* infection. Previously, Chan et al. (1993) indicated co-incubation of potato suspension cells (PSC) with *Agrobacterium* significantly improved the transformation efficiency of rice. PSC was rich in acetosyringone and sinaptic acid, which were generally believed to enhance transformation (Czernilofsky et al., 1986).

Since acetosyringone in the co-cultivation medium is known to enhance T-DNA strand synthesis (Li et al., 1992) and T-strand production is reported to peak 12-24 hours after acetosyringone-mediated induction (Culianez-Macia and Hepburn 1988), induction of *vir* genes during co-cultivation and efficient release of T-DNA may be the cause of high frequency transformation.

Use of exogenous acetosyringone has enhanced transformation efficiency in certain dicots, and has been found to be one of the factors affecting transformation in monocots. Rashid et al. (1996) reported effect of acetosyringone on transformation efficiency evaluated by number of GUS-expressing calli in Basmati 370. No transformation efficiency was obtained when acetosyringone was omitted from the medium. The calli turned brown and died during selection.

Khanna and Raina (1999) reported the effect of acetosyringone concentration in preinduction and co-cultivation media of *indica* rice (IR-43 and Basmati cultivar Karnal Local). Its total absence, from the co-cultivation as well as preinduction media, resulted in complete absence of GUS expression. Increasing the levels of acetosyringone from 60 to 200 μM in the co-cultivation medium did not produce any significant enhancement of GUS expression in any explant of the cultivars tested.

In this study, 100 μM of acetosyringone was shown to be sufficient. Both *A. tumefaciens* strain EHA105 (pCAMBIA1301), EHA105 (pCAMBIA5305) and EHA105 (pCAMBIA1306IC) provided successful results. This EHA105 is an efficient strain for transformation of higher plants containing a disarmed version of pTiBo542 (Hood et al., 1986). The strains that carried pTiBo542, a super-virulent Ti plasmid, were reported to operate very efficiently in transformation (Komari, 1989).

GUS expression in the callus stage was examined immediately after 3 days of co-cultivation with *A. tumefaciens*. Expression of GUS in rice callus was obtained at 15.79%. This was similar to the report of Lee et al. (1999) which transform 13 cultivars (including Chorungbyeo, Dongjinbyeo, Hwayoungbyeo, IR43, Milyang 23, Milyang 99, Milyang 117, Nagdongbyeo, Nongnbyeo, Palgongbyeo, Samgangbyeo, Sangjubyeo, and Sinseonchalbyeo) of rice with *A. tumefaciens* LBA4404 (pTOK233) carrying genes for kanamycin resistance (*npt*II), hygromycin resistance (*hpt*) as selectable marker and GUS (*uidA*) as a reporter gene.

GUS expression in the callus stage was examined after 8 weeks on selection medium. Calli co-cultivated with *A. tumefaciens* EHA105 (pCAMBIA1301) showed the recovery of hygromycin resistant calli that express GUS activity at 19.4%. Calli co-cultivated with *A. tumefaciens* EHA105 (pCAMBIA5305) showed the recovery of hygromycin resistant calli that express GFP expression at 17.4%. Calli co-cultivated with *A. tumefaciens* EHA105 (pCAMBIA1306IC) showed the recovery of hygromycin resistant calli that express GFP expression and GUS activity at 16.4%. This was similar to the report of Hiei et al.(1994) reported the frequency of hygromycin resistant calli that express GUS activity at 23%.

Transformation efficiency obtained in this work determined by plants that stably expressed GFP fluorescence (for pCAMBIA5305) and both GFP fluorescence and GUS activity (for pCAMBIA1306IC) relative to the number of pieces of scutellum-derived calli that had been co-cultivated with bacterial cells was 9.8% and 8.2% which has been as high as those reported for rice (Hiei et al., 1994, Khanna and Raina, 1999, Zhang et al., 1997 and Rashid et al., 1996). Furthermore, the majority of plants produced by this method were free of morphological aberrations, probably as a result of the fact that the cells were maintained in culture *in vitro* for a short time. Plants were obtained in only five to six months after transformation. This is a considerable advantage over protoplast systems that often require twice this amount of time.

Hiei et al. (1994) reported *Agrobacterium*-mediated transformation of *japonica* rice, Tsukinohikari, Asanohikari and Koshihikari using scutellum-derived

calli as starting material. *A. tumefaciens* strain LBA4404 (pTOK233), haboring genes for kanamycin resistance (*npt*II), hygromycin resistance (*hpt*) as selectable marker and GUS (*uidA*) as a reporter gene, was used in transformation. Transformation efficiency determined by hygromycin-resistant plants that stably expressed GUS activity relative to the number of pieces of scutellum-derived calli that had been co-cultivated with bacterial cells was range between 10-30%.

Zhang et al. (1997) reported the transformation of calli induced from embryos of mature seeds with *A. tumefaciens* strain LBA4404 carrying the plasmid pTOK233. *indica* rice cultivar, Pusa Basmati 1 and the *japonica* cultivars, E-yi 105, E-wan 5, and Zhong-shu-wan-geng (ZSWG), was used as targets for *Agrobacterium*-mediated transformation. Transformation frequencies were 13.5%, 13.0%, 9.1% and 9.3%, respectively.

Rashid et al. (1996) reported *Agrobacterium*-mediated transformation of *indica* rice, Basmati 370, Basmati 385 and Basmati 6129. The scutellum-derived calli were co-cultivated with *A. tumefaciens* strain EHA101 (pIG121Hm), carrying *hpt* as a selectable marker and GUS as a reporter gene. Basmati 370 showed a transformation frequency of 22%, Basmati 385 showed a transformation frequency of 4.8% whereas Basmati 6129 fell to form plantlet and no regenerant were obtained.

Khanna and Raina (1999) reported *Agrobacterium*-mediated transformation system in *indica* cultivars (IR-64 and Basmati cultivar Karnal Local). *Agrobacterium* stain AGL1, carrying the hypervirulence region of pTiBo542, was tested in combination with an ordinary binary vector, pCAMBIA1301. Moreover, *Agrobacterium* stain LBA4404 (pTOK233) was also used. Transformation frequency was approximate 4-5%.

These indicated that the frequency of transformation varies with both plant genotype and with the transformation system used. In general, *japonica* cultivars of rice are more tissue culture-responsive and give higher frequency of transformation than *indica* cultivars, and one would find variations even between different *japonica* cultivars.

A total of 14 plants could be regenerated from GFP-positive and hygromycin-resistant calli of KDML 105. Of these, 9 were from calli transformed with EHA105 (pCAMBIA5305) and 5 were from calli transformed with EHA105 (pCAMBIA1306IC). In PCR assays using specific primers derived from the GFP coding sequence, genomic DNA from all 14 regenerants showed the expected size of the amplified DNA fragment, indicating that these plants are true transgenic plants. Since *npt*II is present outside the T-DNA in pCAMBIA5305 and pCAMBIA1306IC, *npt*II gene specific primers were also used to check for the presence of any contaminating *Agrobacterium* cells in the plant tissues. None of the regenerants analysed showed amplification of sequences beyond the T-DNA border. This indicates that the tissues were completely cured of *Agrobacterium*. The results were similar to the studies of Khanna and Raina (1999).

Khanna and Raina (1999) reported PCR analyses of putative transformants resulting from infections with AGL1 (pCAMBIA1301). Genomic DNA of IR-64 and KL cultivars was amplified with *hpt* gene primers as well as *npt*II gene primers. Since *npt*II is present outside the T-DNA in pCAMBIA1301, *npt*II gene specific primers were also used to check for the presence of any contaminating *Agrobacterium* cells in the plant tissues. Twenty-four out of 27 plants showed amplification of *hpt* gene sequences and no bands were detected from *npt*II gene primers in all plants.

More recently, Upadhyaya et al. (2000) reported *A. tumefaciens* strain AGL1-mediated transformation of calli derived from mature embryos of Australian rice cultivars, Jarrah and Amaroo. The presence of regions outside the T-DNA border sequences was observed in 7 transgenic lines by using binary vector-backbone specific primers. Moreover, frequent co-linear long transfers of DNA inclusive of the whole binary vector have been observed previously by Wenck et al. (1997).

A recent study, Chiu et al. (1996) found that a re-engineered green fluorescent protein gene (sGFP) with the preferred codon usage of human protein under a strong constitutive promoter (35SC4PPDK) gave 20-fold higher GFP expression in maize leaf cells than original jellyfish GFP sequence. This promoter was constructed by fusing the 35S enhancer to the basal promoter and $5^{/}$ untranslated region of the maize

C4PPDK gene; a universal transcription enhancer. In this study, the expression of GFP reporter gene under control of the CaMV35S promoter from the transformation vectors, pCAMBIA5305 and pCAMBIA1306IC was detected by fluorescent microscopy.

GFP expression was detected in all of the tissues of transgenic rice plants that were examined. Analysis of putative transformed rice embryogenic calli was performed after selection for 8 weeks. The transformed calli were exhibited very bright-green fluorescent when observed under fluorescent microscope. Although weak yellowish or greenish fluorescence occurred in control calli, the identification of GFP-expressing calli was unambiguous by using of FITC filter alleviates this problem as reported previously by Pang et al. (1996).

Moreover, when GFP transformed calli were illuminated with a hand-held, long-wave UV lamp, fluorescence was readily detectable by eye which similar to the results of Vain et al., 1998. This provided the inexpensive system to detection of GFP fluorescence. However, due to its broad-wavelength range and its wide lighty-diffusion angle, the hand-held lamp produced only limited energy in the wavelength range useful for GFP excitation. Therefore, this system only allowed identification of rice tissues expressing GFP at high levels.

Transgenic rice plants were regenerated from visually selected GFP-positive calli. The high levels of GFP fluorescence could be observed primarily in the root system. Conversely, under all conditions, GFP-positive tissue was more readily identified in calli and the young leaf primordia of shoot tips than in older, more developed parts of regenerating shoots. Expression was limited in chlorophytic tissues, such as developed leaves. This was similar to the result of Molinier et al. (2000). As development progressed, chlorophyll autofluorescence increasingly masked the GFP fluorescence which thus became more difficult to detect in tissues comprising more differentiated cells, exactly as previously observed with the primary transformants. In this results, GFP was most clearly detectable in the vascular bundle of the rice tissues. This is in agreement with the report of Benfey et al. (1989) and

Jefferson et al. (1987) as described previously that the CaMV 35S promoter is more strongly expressed in cells of the vascular bundle.

Molinier et al. (2000) reported the use of GFP as a visual selection marker for transformed tobacco tissue. The higher cytoplasmic density in young tissues may explain why GFP was detected more easily there than in older leaves where the vacuole, devoid of GFP, constitutes the largest part of the cell.

Vain et al. (1998) reported rice transformation system using particle bombardment of immature embryos. The *mgfp4* gene under the control of the 35S promoter produced bright-green fluorescence easily detectable and screenable in rice calli. Various techniques to observed GFP was described. However, the inability to visualize GFP expression in regenerated plants may be due to the limited strength of the CaMV35S promoter in rice might be responsible for the low apparent expression of GFP in rice leaf tissue.

Sheen et al. (1995) reported either a strong constitutive (35SC4PPDK) or a heat-shock promoter can direct the expression of GFP which is easily detectable in maize mesophyll protoplasts. In this single-cell system, bright green fluorescence emitted from GFP is visible when excited with UV or blue light even in the presence of blue fluorescence from the vacuole or the red chlorophyll autofluorescence from chloroplasts using a fluorescence microscope. The bright yellow regions were observed when the red and green fluorescence overlapped. The majority of GFP was located in the cytoplasm and some was concentrated around the nucleus. A high level of GFP expression is critical for the visualization because a similar construct with the 35S promoter (15-fold weaker than the hybrid promoter) gave very faint green fluorescence.

From this study, GFP was concluded to be more superior to GUS for high magnification microscopic studies. GFP is fairly stable, can be used in living cells and does not require substrates or any other chemicals to visualize. Furthermore, handling and analysis of the biological material does not require fixing or staining. However, GFP production levels in the cell were too low to be visualized in UV light with low

magnification imaging. For macroscopic studies, the GUS reporter system was superior, since the blue stain was visualized easily with the naked eye. Microscopic studies with the GUS transformants were less successful than the studies with the GFP-marked stains, since the blue staining was difficult to visualize and a large proportion of the transformants showed secretion of the enzyme or leakage of the blue crystals from the cells.

Quaedvlieg et al. (1998) have combined the advantages of GFP and GUS by constructing bifunctional reporter genes marking use of the fact that both GFP and GUS can tolerate N-or C-protein fusions. This approach makes it possible to combine the advantage of GUS, its high sensitivity in histochemical staining, with the advantages of GFP as a vital marker. The fusion proteins were functional in transient expression studies in tobacco using DNA bombardment, and in stably transformed *Arabidopsis thalina* and *Lotus japonicus* plants. The results show that high level of expression does not interfere with efficient stable transformation in *A. thaliana* and *L. japonicus.*

In this study, the combination of the genes encoding GFP and GUS reporter in a binary vector are studied for use in transformation of rice plants. The transgenic nature of rice plants (transformed with pCAMBIA1306IC) was confirmed by using the PCR technique and detection of GFP fluorescence as well as histochemical assay of GUS reporter gene. The results show that the detected GFP expression is completely correlated with GUS activity in all transformed rice plants. The PCR analysis showed expected band at 720 bp suggested that GFP gene was present in all transformants.

Although, histochemical staining of GUS activity in calli, roots of transformant transformed with pCAMBIA1306IC showed uniformed blue staining, the leaves segments showed blue staining at only cut surface. This result was similar to the studies of Dong et al. (1996) which determined that this resulted from limited penetration of the X-Gluc substrate.

The advantage of assaying for the presence to the foreign gene in transgenic tissues with PCR is that a positive results can signify that the full sequence is present (as well as being a quick test). On the other hand, information of the number and site of integration is obtainable only with the Southern blotting approach. Therefore, a further study may be concentrated on transformed status by estimation of copy number and site of integration by Southern blot analysis.

In summary, an efficient *Agobacterium*-mediated transformation of *indica* rice cultivars (KDML 105) was developed to produce transgenic lines with stable transgene expression. The transformed plants appeared to be morphologically identical to nontransformed, control plants. CaMV35S promoter was demonstrated to be used for transgene expression in rice. This technique will allow the genetic improvement of diverse varieties of rice, as well as many aspects of the molecular biology. Experiments are underway to utilize these procedures to study the expression of various genes driven by various promoters in rice, regulation of gene expression, funtional analysis of genes, gene tagging and introduction of agronomically useful gene into rice. This technology provides a means to improve traits in rice. Therefore, *Agrobacterium*-mediated gene transfer is now available as an alternative routine and straightforward method for the genetic modification of rice.

The use of GFP as a screenable marker demonstrates how a new transformation system can be established in the absence of a herbicide-or antibiotic-based selection system. GFP has been particularly advantageous in detecting low-frequency transformation events with confidence, enabling the early discard of non-transformed material. GFP will play an essential role in removing the gene transfer constraint from fundamental science and also in applied programmes involving genetic engineering.

# CHAPTER V

# CONCLUSIONS AND RECOMMENDATIONS

1. *Agrobacterium*-mediated transformation can be used to transformed green fluorescent protein (GFP) reporter gene into *indica* rice variety (*O. sativa* cv. KDML 105) using EHA105 (pCAMBIA5305) and EHA105 (pCAMBIA1306IC).

2. Embryogenic callus induction was compared between 2NB and 2MS medium. 2NB medium gave 79.2±3.4% embryogenic callus induction, higher than 2MS medium (54.6±2.5%).

3. Plantlet regeneration was compared among 4 regeneration media; MS1-RE, NB1-RE, NB2-RE and NB4-RE. High percentage (68.2±13.6%) was obtained from NB4-RE generation medium.

4. Transformation efficiency of callus co-cultivated with EHA105 (pCAMBIA1301) for 3 days revealed by GUS assay was 15.8% and for 8 weeks was 19.4%.

5. Hygromycin resistant calli from pCAMBIA1301, pCAMBIA5305 and pCAMBIA1306IC were obtained after 4 weeks on selection medium (50 mg/l hygromycin). The uninoculated calli did not show continuous growth, turned brown and died in selection medium.

6. The transgenic nature of putative transformed rice plants was confirmed by detection of GFP fluorescence and PCR analysis revealed all of them were true transgenic plants.

7. The transformation frequency obtained in this work determined by plants that stably express GFP for pCAMBIA5305 and both GFP fluorescent and GUS activity for pCAMBIA1306IC was 9.8% and 8.2%, respectively.

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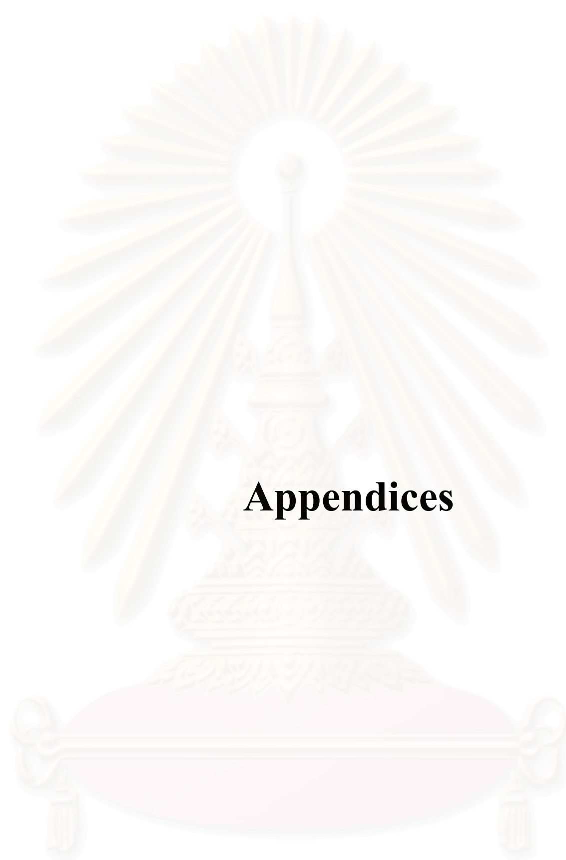

# Appendices

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# Appendix A

The composition of rice tissue culture and *Agrobacterium tumefaciens* medium

**Table 1.** The composition of NB medium (Li et al. 1993)

| Solution | Chemicals | Concentration (mg/l) |
|---|---|---|
| N6 Macronutrients | $KNO_3$ | 2,830 |
| | $(NH_4)_2SO_4$ | 463 |
| | $CaCl_2.2H_2O$ | 166 |
| | $MgSO_4.7H_2O$ | 185 |
| | $KH_2PO_4$ | 460 |
| B5 micronutrients | KI | 0.75 |
| | $H_3BO_3$ | 3 |
| | $CoCl_2.6H_2O$ | 0.025 |
| | $MnSO_4.7H_2O$ | 10 |
| | $ZnSO_4.7H_2O$ | 2 |
| | $Na_2MoO_4.7H_2O$ | 0.25 |
| | $CuSO_4.5H_2O$ | 0.025 |
| FeEDTA | $FeSO_4.7H_2O$ | 27.8 |
| | $Na_2EDTA.2H_2O$ | 37.8 |
| B5 vitamins | Myo-inositol | 100 |
| | Nicotinic acid | 1 |
| | Pyridoxine HCl | 1 |
| | Thiamine HCl | 10 |
| | Casein hydrolysate | 300 |
| | L-Proline | 500 |
| | L-Glutamine | 500 |
| | Sucrose | 30,000 |
| | Agar | 8,000 |

pH 5.8

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**Table 2.** The composition of 2NB medium (callus induction medium)

| Solution | Chemicals | Concentration (mg/l) |
|---|---|---|
| N6 Macronutrients | $KNO_3$ | 2,830 |
| | $(NH_4)_2SO_4$ | 463 |
| | $CaCl_2.2H_2O$ | 166 |
| | $MgSO_4.7H_2O$ | 185 |
| | $KH_2PO_4$ | 460 |
| B5 micronutrients | KI | 0.75 |
| | $H_3BO_3$ | 3 |
| | $CoCl_2.6H_2O$ | 0.025 |
| | $MnSO_4.7H_2O$ | 10 |
| | $ZnSO_4.7H_2O$ | 2 |
| | $Na_2MoO_4.7H_2O$ | 0.25 |
| | $CuSO_4.5H_2O$ | 0.025 |
| FeEDTA | $FeSO_4.7H_2O$ | 27.8 |
| | $Na_2EDTA.2H_2O$ | 37.8 |
| B5 vitamins | Myo-inositol | 100 |
| | Nicotinic acid | 1 |
| | Pyridoxine HCl | 1 |
| | Thiamine HCl | 10 |
| | 2,4-D | 2 |
| | Casein hydrolysate | 300 |
| | L-Proline | 500 |
| | L-Glutamine | 500 |
| | Sucrose | 30,000 |
| | Agar | 8,000 |

pH 5.8

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**Table 3.** The composition of 2NB-AS medium (co-cultivation medium)

2NB media supplemented with

| Chemicals | Concentration (g/l) |
| --- | --- |
| Sucrose | 30 |
| Glucose | 10 |

Acetosyringone 100 µM (add after autoclave)

pH 5.2

**Table 4.** The composition of 2NB-CH medium (selection medium)

2NB medium autoclave cool to room temperature and add

| Chemicals | Concentration (mg/l) |
| --- | --- |
| Cefotaxime | 500 |
| Hygromycin | 50 |

pH 5.2

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**Table 5.** The composition of NB-RE medium (regeneration medium)

| Solution | Chemicals | Concentration (mg/l) |
|---|---|---|
| N6 Macronutrients | $KNO_3$ | 2,830 |
| | $(NH_4)_2SO_4$ | 463 |
| | $CaCl_2.2H_2O$ | 166 |
| | $MgSO_4.7H_2O$ | 185 |
| | $KH_2PO_4$ | 460 |
| B5 micronutrients | KI | 0.75 |
| | $H_3BO_3$ | 3 |
| | $CoCl_2.6H_2O$ | 0.025 |
| | $MnSO_4.7H_2O$ | 10 |
| | $ZnSO_4.7H_2O$ | 2 |
| | $Na_2MoO_4.7H_2O$ | 0.25 |
| | $CuSO_4.5H_2O$ | 0.025 |
| FeEDTA | $FeSO_4.7H_2O$ | 27.8 |
| | $Na_2EDTA.2H_2O$ | 37.8 |
| B5 vitamins | Myo-inositol | 100 |
| | Nicotinic acid | 1 |
| | Pyridoxine HCl | 1 |
| | Thiamine HCl | 10 |
| | L-Proline | 500 |
| | L-Glutamine | 500 |
| | Sucrose | 30,000 |
| | Yeast extract | 1,000 |
| | Casein hydrolysate | 300 |
| | Agar | 16,000 |

Coconut water 15%

pH 5.8

**Table 6.** The composition of NB1-RE, NB2-RE and NB4-RE regeneration medium

NB-RE regeneration medium supplemented with

| Medium | Concentration of BAP (mg/l) |
|---|---|
| NB1-RE | 1 |
| NB2-RE | 2 |
| NB4-RE | 4 |

pH 5.8

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**Table 7.** The composition of MS medium (Murashige and Skoog, 1962)

| **Solution** | **Chemicals** | **Concentration (mg/l)** |
|---|---|---|
| Macronutrients | $NH_4NO_3$ | 1,650 |
| | $KNO_3$ | 1,900 |
| | $CaCl_2.2H_2O$ | 440 |
| | $KH_2PO_4$ | 170 |
| | $MgSO_4.7H_2O$ | 370 |
| Micronutrients | KI | 0.83 |
| | $H_3BO_3$ | 6.2 |
| | $CoCl_2.6H_2O$ | 0.025 |
| | $MnSO_4.H_2O$ | 16.9 |
| | $ZnSO_4.7H_2O$ | 8.6 |
| | $Na_2MoO_4.2H_2O$ | 0.25 |
| | $CuSO_4.5H_2O$ | 0.025 |
| FeEDTA | $FeSO_4.7H_2O$ | 27.8 |
| | $Na_2EDTA.2H_2O$ | 37.8 |
| MS vitamins | Myo-inositol | 100 |
| | Nicotinic acid | 0.5 |
| | Pyridoxine HCl | 0.5 |
| | Thiamine HCl | 0.1 |
| | Glycine | 2 |
| | Sucrose | 30,000 |
| | Agar | 8,000 |

pH 5.8

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**Table 8.** The composition of 2MS medium

| Solution | Chemicals | Concentration (mg/l) |
|---|---|---|
| Macronutrients | $NH_4NO_3$ | 1,650 |
| | $KNO_3$ | 1,900 |
| | $CaCl_2.2H_2O$ | 440 |
| | $KH_2PO_4$ | 170 |
| | $MgSO_4.7H_2O$ | 370 |
| Micronutrients | KI | 0.83 |
| | $H_3BO_3$ | 6.2 |
| | $CoCl_2.6H_2O$ | 0.025 |
| | $MnSO_4.H_2O$ | 16.9 |
| | $ZnSO_4.7H_2O$ | 8.6 |
| | $Na_2MoO_4.2H_2O$ | 0.25 |
| | $CuSO_4.5H_2O$ | 0.025 |
| FeEDTA | $FeSO_4.7H_2O$ | 27.8 |
| | $Na_2EDTA.2H_2O$ | 37.8 |
| MS vitamins | Myo-inositol | 100 |
| | Nicotinic acid | 0.5 |
| | Pyridoxine HCl | 0.5 |
| | Thiamine HCl | 0.1 |
| | Glycine | 2 |
| | 2,4-D | 2 |
| | Sucrose | 30,000 |
| | Agar | 8,000 |

pH 5.8

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**Table 9.** The composition of MS1-RE medium

| Solution | Chemicals | Concentration (mg/l) |
|---|---|---|
| Macronutrients | $NH_4NO_3$ | 1,650 |
| | $KNO_3$ | 1,900 |
| | $CaCl_2.2H_2O$ | 440 |
| | $KH_2PO_4$ | 170 |
| | $MgSO_4.7H_2O$ | 370 |
| Micronutrients | KI | 0.83 |
| | $H_3BO_3$ | 6.2 |
| | $CoCl_2.6H_2O$ | 0.025 |
| | $MnSO_4.H_2O$ | 16.9 |
| | $ZnSO_4.7H_2O$ | 8.6 |
| | $Na_2MoO_4.2H_2O$ | 0.25 |
| | $CuSO_4.5H_2O$ | 0.025 |
| FeEDTA | $FeSO_4.7H_2O$ | 27.8 |
| | $Na_2EDTA.2H_2O$ | 37.8 |
| MS vitamins | Myo-inositol | 100 |
| | Nicotinic acid | 0.5 |
| | Pyridoxine HCl | 0.5 |
| | Thiamine HCl | 0.1 |
| | Glycine | 2 |
| | BAP | 1 |
| | Sucrose | 30,000 |
| | Agar | 16,000 |

pH 5.8

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**Table 10.** The composition of AB medium (Chilton et al., 1974)

| Solution | Chemicals | Concentration (mg/l) |
|---|---|---|
| AB buffer | $K_2HPO_4$ | 1,500 |
| | $NaH_2PO_4$ | 200 |
| AB salt | $NH_4Cl$ | 1000 |
| | $MgSO_4.7H_2O$ | 300 |
| | KCl | 150 |
| | $CaCl_2.2H_2O$ | 150 |
| | $FeSO_4.7H_2O$ | 2.5 |
| | Glucose | 5,000 |
| | Agar | 15,000 |

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**Table 11.** The composition of AAM medium (Toriyama and Hinata, 1985)

| Solution | Chemicals | Concentration (mg/l) |
|---|---|---|
| AA macronutrients | $Na_2HPO_4.2H_2O$ | 169.6 |
| | $MgSO_4.7H_2O$ | 500 |
| | KCl | 150 |
| | $CaCl_2.2H_2O$ | 150 |
| AA micronutrients | $MnSO_4.4H_2O$ | 10 |
| | $Na_2MoO_4.2H_2O$ | 0.25 |
| | $H_3BO_3$ | 3.0 |
| | $ZnSO_4.7H_2O$ | 2.0 |
| | $CuSO_4.5H_2O$ | 0.0387 |
| | $CoCl_2.6H_2O$ | 0.025 |
| | KI | 0.75 |
| AA iron | $FeSO_4.7H_2O$ | 28 |
| MS vitamin | Inositol | 100 |
| | Nicotinic acid | 0.5 |
| | Pyridoxine HCl | 0.5 |
| | Thiamine HCl | 0.5 |
| AA amino acid | Glycine | 7.5 |
| | Arginine | 174 |
| | Glutamine | 876 |
| | Casamino acid | 500 |
| | Sucrose | 68,500 |
| | Glucose | 35,000 |

Acetosyringone 100 μM (add after autoclave)

pH 5.2

**Table 12.** The preparation of chemicals used in tissue culture

| Chemicals | Preparation |
|---|---|
| 2,4-Dichlorophenoxyacetic acid) (2,4-D) (1 mg/ml) | Dissolve 100 mg of 2,4-D in 1 ml absolute ethanol, add 3 ml of 1 N KOH, adjust to pH 6.0 with 1 N HCl (very sensitive adjust carefully), and make upto 100 ml with sterilize deionized water. |
| 6-Benzylaminopurine (BAP) (2 mg/ml) | Weigh 100 mg BAP, add 1 N KOH dropwise until powder is dissolved, make upto 50 ml with sterilize deionized water. |
| AS- Acetosyringone (100 μM) | Add 19.62 mg of acetosyringone to 1 ml of Dimethyl sulphoxide (DMSO) or methanol. Protect from light and add to media after autoclaving. |

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# Appendix B

**β-Glucuronidase (GUS) assays** (Jefferson, 1987)

GUS extraction buffer:

50 mM sodium phosphate buffer , pH 7.0

10 mM 2-mercaptoethanol

10 mM $Na_2EDTA$

0.1% sodium N-laurylsarcosine

0.1% Triton X-100

Histochemical staining buffer (X-Gluc):

To prepare a stock solution, 100 mg X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) is dissolved in 1 ml dimethylformamide.

Standard staining solution:

Add 50 μl X-Gluc stock solution to 10 ml GUS extraction buffer (final concentration is 0.5 mg/ml).

Enhanced staining solution (optional):

Mix 50 μl X-Gluc stock solution with 9.8 ml GUS extraction buffer, 100 μl 50 mM $K_3Fe(CN)_6$ and 100 μl 50 mM $K_4Fe(CN)_6$.

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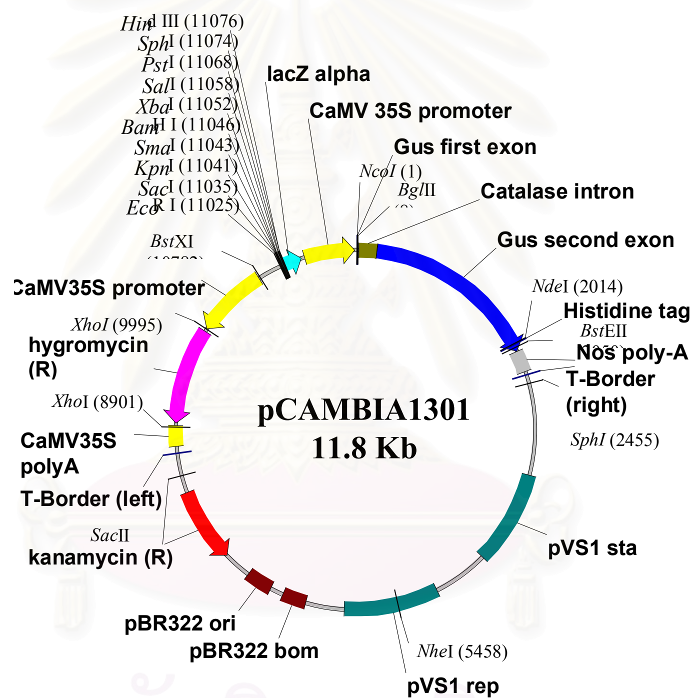
# Appendix C

Restriction mapping of plasmid pCAMBIA1301 (C.1), pCAMBIA5305 (C.2) and pCAMBIA1306IC (C.3)

(C.1) pCAMBIA1301

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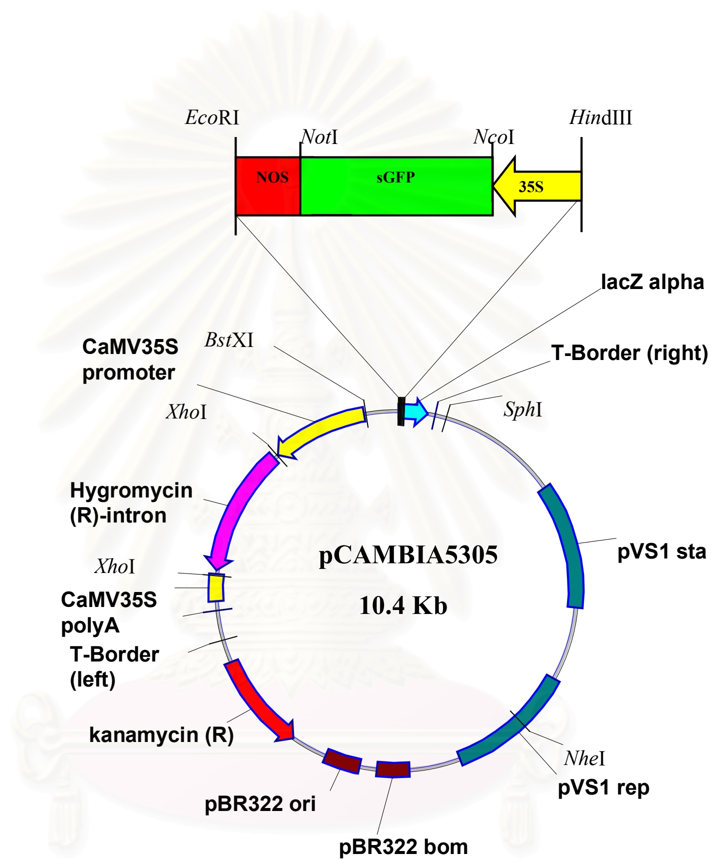
(C.2) pCAMBIA5305

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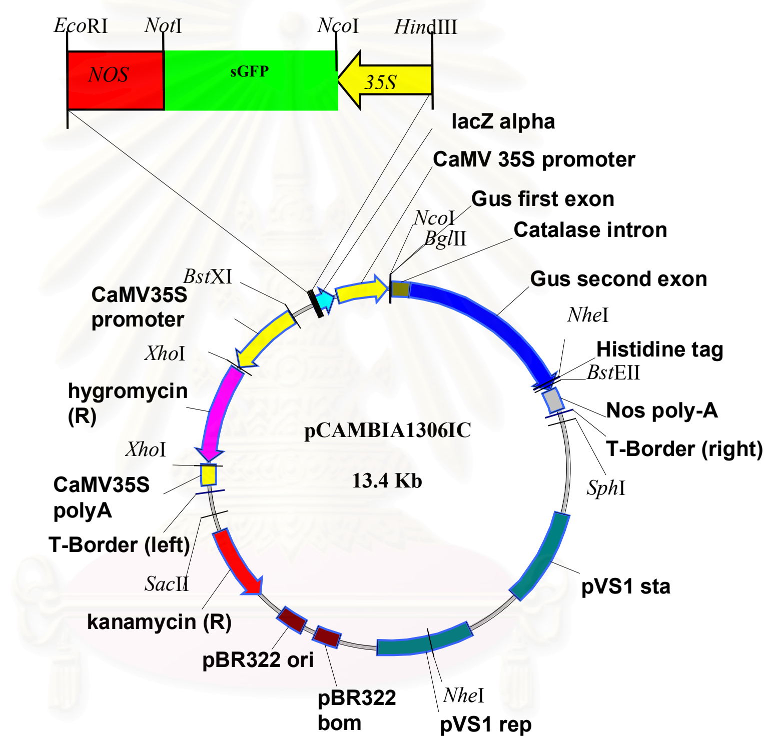
(C.3) pCAMBIA1306IC

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# Biography

Miss Chanprapa Imjongjirak was born on May 3, 1976 in Bangkok, Thailand. She graduated with the degree of Bachelor of Science in Biology from the department of Botany, Kasetsart University in 1996. In 1997, she enrolled in Master degree of Science at the department of Biochemistry, Chulalongkorn University.

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