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Selection of cell lines with high DAPI stainability from rice (*Oryza sativa* L.) suspension culture for protoplast isolation and regeneration

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Abstract. Suspension culture plays an important role in high yield of protoplast isolation. From our routine maintenance of rice suspension lines, we found growth rates of most suspension lines were not quite stable even after subculture for 3-4 months. A DNA specific binding fluorochrome-DAPI(4',6-diamidino-2-phenylindole) was used to elucidate the nuclear status of cell lines during culture. Suspension lines derived from anther calli were used for this study. The results show that cell lines tend to have differential DAPI stainability during culture. The DAPI stainability is classified into high, medium and low groups based on percentage of suspension cells exhibiting nuclear fluorescence immediately after staining. Variations in regeneration ability and protoplast yield exist among three groups. High DAPI stainability cell lines tend to have higher regeneration ability and higher protoplast yields than the others. The average protoplast yields of high DAPI stainability lines are 2.5×10^7 protoplasts/g • fresh wt. cells and 7.8×10^6 protoplasts/g • fresh wt. cells for cultivars Hsin-Chu 56 (HC56) and Taipei 309 (TP309) respectively. A maximum yield up to $2\text{-}3 \times 10^8$ protoplasts/g • fresh wt. cells was also noted occassionally. Plating efficiency apparently increased when selection for high DAPI stainability and medium size of nucleus were applied. The non-stained cells were believed to be non-vital or aged cells in a contrast study on FDA test.

Key words: Anther culture; DAPI(4',6-diamidino-2-phenylindole); FDA(fluorescein diacetate); Fluorescence; Protoplast regeneration; Suspension culture.

Introduction

Protoplast isolation and regeneration in rice (*Oryza sativa* L.) is no longer unreachable during the past few years (Fujimura *et al.*, 1985; Yamada *et al.*, 1986; Toriyama *et al.*, 1986; Abdullah *et al.*, 1986; Kyozuka *et al.*, 1987; Kyozuka *et al.*, 1988; Lee *et al.*, 1989; Jenes and Pauk, 1989; Liu *et al.*, 1989; Li and Murai, 1990). Achievements in gene manipulation through protoplast culture in rice have been demonstrated (Uchimiya *et al.*, 1986; Zhang *et al.*, 1988; Toriyama *et al.*, 1988; Datta *et al.*, 1990). Genetic manipulation of haploid protoplast in comparison with diploid was believed to be more valuable for the expression of incorporated gene (Toriyama *et al.*, 1986).

The essential points for success in rice protoplast regeneration can be referred to factors such as: (1) cultural media (Yamada *et al.*, 1986; Toriyama *et al.*, 1986; Lee *et al.*, 1989; Li and Murai, 1990); (2) selection of embryogenic calli for initiation of suspension culture (Datta *et al.*, 1990); (3) establishing fast growing cell lines (Thompson *et al.*, 1986); (4) conditions of enzyme solution for protoplast isolation (Lee *et al.*, 1989); (5) protoplast plating on agarose beads or block; (6) the utilization of nurse cells (Abdullah *et al.*, 1986; Kyozu-

ka *et al.*, 1987); (7) genotypes (Yamada *et al.*, 1986; Kyozuka *et al.*, 1988; Jenes and Pauk, 1989); and (8) age of suspension lines etc.. However, our studies indicated that establishing a stable embryogenic suspension culture is not easy for an inexperienced person and non-selected suspension culture tends to show variations in growth rate during subcultures. Even fast growing cell lines derived from embryogenic calli were established, growth vigor declined after subculture for a period of time. Growth pattern of cell culture is a very complex issue and tends to vary from material to material as well as cultural methodologies (King and Street, 1977). Suspension lines good for protoplast isolation and regeneration can vary from one month (Li and Murai, 1990) to one year (Jenes and Pauk, 1989; Datta *et al.*, 1990). Protoplast yields generally varied from experiment to experiment. The reason why these differences exist remains unclear. DAPI(4',6 - diamidino - 2 - phenylindole) is a DNA-specific fluorochrome and has been used as a vital stain (Coleman and Goff, 1985). In an attempt to determine the nuclear status in suspension cultures of different growth rate, differential stainability of DAPI on culture lines derived from the same origin and same culture period were shown. In this communication, we report the effects of DAPI stainability of suspension lines on rice protoplast isolation and regeneration.

Materials and Methods

Plant Materials and Induction of Callus from Anther Culture

Two cultivars, Taipei 309 and Hsin-Chu 56, were used in this study. Field materials were sampled from Chai-Yi Experimental Station of Taiwan Agricultural Research Institute. Primary calli derived from anther culture were used for establishing the suspension cultures for this study. Principal procedures for anther culture followed that of Chen and Chen (1979) and Chen *et al.* (1982) with slight modification. Rice culms with unemerged young rice inflorescences were sampled at the stage when the distance between ligules of the flag leaf and the next leaf was about 3 to 5 cm. The excised culms were pretreated at 8°C for 10-14 days. Pretreated culms were surface sterilized with 70% ethanol and torn off the outmost leaflet followed by two changes of 0.5% sodium hypochlorite, each for two minutes and then rinsed with sterilized distilled water. Anthers dissected from panicles of the center one third of inflorescence were inoculated onto D medium (Chen *et al.*, 1982), which is composed of N6 (Chu *et al.*, 1975) inorganic elements, MS (Murashige and Skoog, 1962) organic components, 4 ppm α-naphthaleneacetic acid (NAA), 2 ppm kinetin and 6% sucrose in liquid medium or agar medium (0.8%) for callus induction. Within 4 to 6 weeks, calli were formed.

Initiation of Suspension Culture from Anther Culture

Calli derived from anther cultures were further transferred into modified AA medium (Thompson *et al.*, 1986) for suspension culture. Suspension was subcultured every 6-8 days. Fast growing lines or suspension lines with differential DAPI stainabilities were subjected to protoplast isolation and plant regeneration test.

Fluorescent and Nuclear Stain of Suspension Cells

For DAPI stainability classifications, samples were taken from cells of suspension lines and stained with 2-3 drops of DAPI stain solution (1.25 μg/ml in McIlvaine's pH 4 buffer with 0.5% Triton X-100 for enhancing penetration) (Coleman and Goff, 1985; Vergne *et al.*, 1987) and checked under the fluorescence microscopy immediately after staining. Percentage of suspension cells exhibiting nuclear fluorescence was estimated from the whole slide. Stainability were roughly grouped into high ($\geqq$ 70%), medium (between 70% and 25%) and low ($\leqq$ 25%). For fluorescence detection, Olympus BH2-RFL fluorescence microscope was used with ultraviolet excitation and 435 nm barrier. Suspension lines with differential stainabilities were selected and subjected to protoplast isolation and regeneration test. Cell viability can be further confirmed by FDA (Fluorescein diacetate) (2 mg in 1 ml acetone diluted with 0.5 M sucrose before use) fluorescence stain (Widholm, 1972) under blue excitation with 495 nm barrier. For ploidy level determination follows that of Chen and Chen (1980) except that modified carbol fuchsin (Kao, 1975) was used as stain.

Protoplast Isolation, Culture and Regeneration

Principal procedures for protoplast isolation and successive cultures followed that of Kyozuka *et al.* (1987) with slight modification. Suspension cell lines, one or two days after subculture, were used for protoplast isolation. Forty to fifty ml suspension cells were

filtrated through 280 μm steel sieve. About one gram fresh weight of the filtrate was mixed with 20-25 ml enzyme solution and incubated at 30 °C for 4-5 h without shaking. Gently pipette the enzyme-protoplast mixture a couple times and filtrate through a series of nylon sieves (140, 45 and 25 μm). Remove the enzyme solution by centrifuge at 800 rpm for 5 min. Wash the protoplasts with KMC solution (Harms and Potrykus, 1978) and pipette out the supernatant after centrifuge (800 rpm, 5 min) for three times. For the final wash, leave the residue volume no more than 1 ml and count the number of protoplasts with hemacytometer. Resuspend protoplast suspension with R2P medium (Ohira *et al.*, 1973) to twice the desired cell density. Mix protoplast suspension with an equal volume of R2P medium containing 2.5% (w/v) agarose (Sea Plaque, LMT, FMC) in petri dish (6 cm). Cut solidified agarose into small blocks and transfer to another 6 cm petri dish containing 5 ml of R2P medium. Add about 1 ml of nurse cells and culture the protoplasts in dark at 27°C. After 10-15 days, remove nurse cells by washing with R2P medium and transfer the agarose block to a new petri dish containing 5 ml of R2P medium. Agarose block containing visible colonies were transferred to N6 medium containing 0.25% (w/v) agarose and incubated under light at 27 °C. Microcalli were picked up and placed on the same medium with a higher agarose concentration (0.5%). Colonies of about 2 mm in diameter were inoculated onto the regeneration medium (N6 with or without kinetin). Shoot and root emerge within one to two months. Plantlets of about 4-5 cm high were grown under hydroponic culture in the growth chamber for another two weeks before transplanting to greenhouse.

Results

Callus Induction From Anther Culture

Microspore calli were induced within 3 to 5 weeks after inoculation on cultural media (Fig. 1A). It is indicated that calli were derived from young microspores but not anther wall or other somatic tissues when anther culture was investigated under the inverted microscope. This is further confirmed by checking the chromosome numbers. The experiment was carried out in two consecutive cropping seasons. Frequencies of anthers forming calli varied from season to season. Varietal difference was also noted. In general, TP309 has an average higher percentage (37.6%) of anthers forming calli than that of HC56 (12.4%). Both D liquid

Table 1. *Frequency of callus induction from rice anther culture*

Cultivar	Culture medium	No. of anther inoculated	No. of anther forming callus	% of anther forming callus	Crop season
TP309	D (l)[a]	117	11	9.4	'88II[b]
	D (s)	58	1	1.7	'88II
	D (l)	172	106	61.6	'89I
	D (s)	126	60	47.6	'89I
	Sub-total	473	178	37.6	
HC56	D (l)	202	24	11.9	'88II
	D (s)	84	4	4.8	'88II
	D (l)	405	63	15.6	'89I
	D (s)	130	11	8.5	'89I
	Sub-total	821	102	12.4	
	Total	1249	280	22.4	

[a](l)- liquid medium, (s)- agar medium.
[b]I- 1st Crop Season, II- 2nd Crop Season.

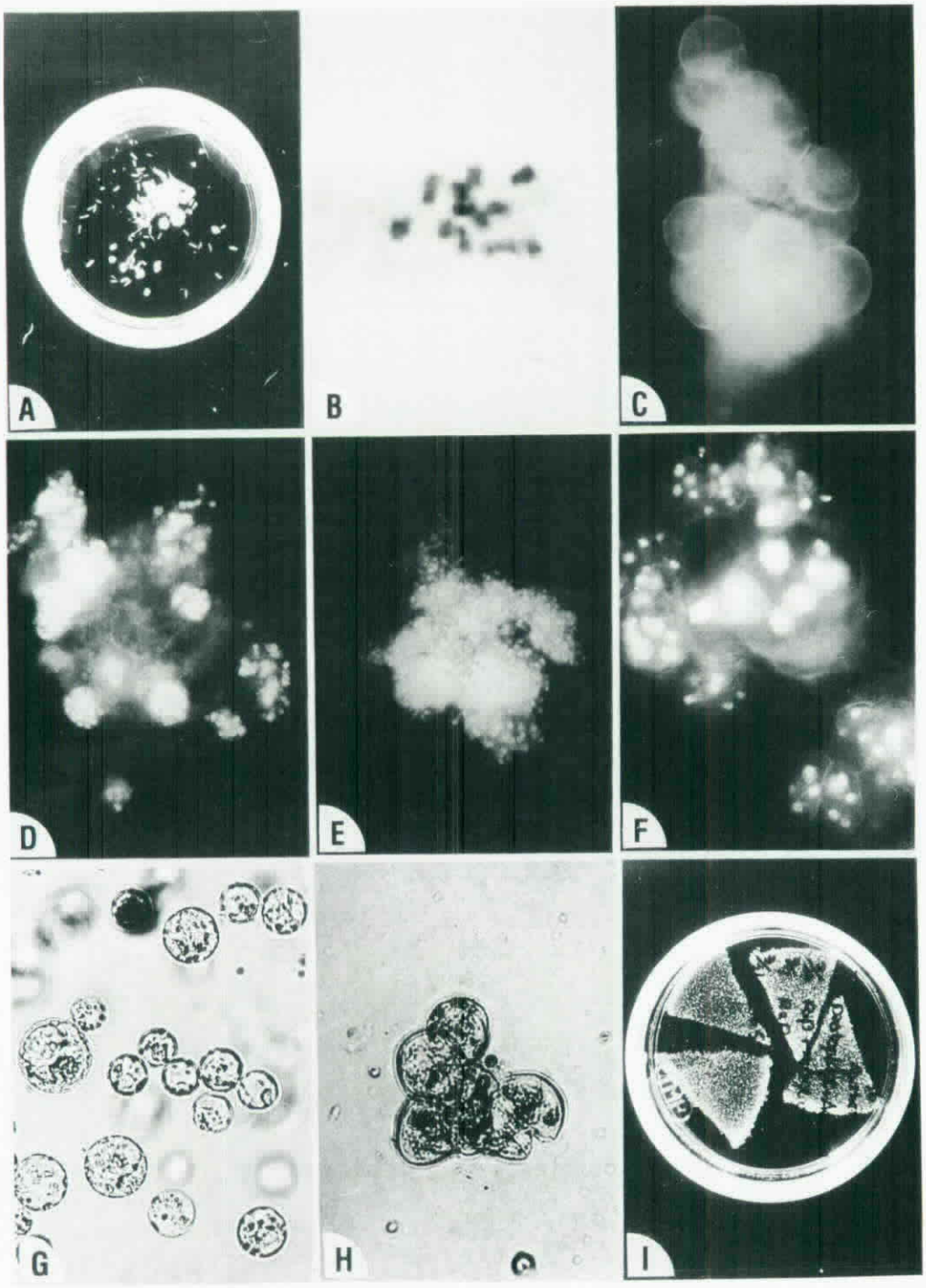

Fig. 1. A, calli derived from anther culture; B, a haploid cell from 6 months old suspension line with chromosome number n = 12, X2500; C – F, calli with differential DAPI stainability under fluorescence microscopy; C, most cells without nuclear fluorescence, X200; D, cell clump with only proportion of cells exhibiting nuclear fluorescence, X160; E, cell clump with most cells exhibiting nuclear fluorescence, X150; F, DAPI stained cells with variations in nuclear size, X300; G, isolated protoplasts, X500 ; H, cell cluster derived from protoplast divisions, X400; I, protoplast-derived colonies in agarose blocks on N6 agarose medium one month after removal of nurse cells.

and agar media were tested. Results showed that callus development was more favored in the liquid medium than in the agar medium (Table 1).

Initiation of Suspension Cultures

Primary calli derived from anther culture were subcultured for another 4 to 6 weeks in D liquid medium or directly put into the AA medium for the initiation of suspension culture. Calli of the same variety were generally pooled together. Growth rate of HC56 is much higher than TP309 when they are subjected to culture in AA medium. Fast growth lines of HC56 can be developed no more than 2 to 3 months, while TP309 takes about 5 to 6 months. Anther calli of TP309 tend to aggregate very easily. Chromosome counts for their ploidy levels were determined when suspensions were 5 to 6 months old. About two thirds of cells were demonstrated haploid (n=12) (Fig. 1B) with most of the rest being diploid. After subculture for more than 6 months, differences in growth rate among cell lines derived from same origin became obvious. DAPI stain was applied on these suspension cell lines. Differential DAPI stainability (Fig. 1C-1E) was observed among cell lines. For cell lines with slow growth rate, suspension calli tend to turn slightly reddish. Poor DAPI stainability (Fig. 1C) was observed in these cell lines. Nuclear size and distribution can be easily viewed by DAPI stain with fluorescence microscopy (Fig. 1C-1F). Some cell lines took up DAPI stain easily, while others either had a slow uptake rate or showed little stainability. Lines with differential DAPI stainability were selected and subjected to protoplast isolation and regeneration test.

Protoplast Isolation and Plant Regeneration from Suspension Lines with Differential DAPI Stainability

It is generally difficult to differentiate the growth rate of suspension cells by their morphological appearance. As shown in Table 2, cell lines are classified into high, medium, and low DAPI stainability. Protoplasts isolated from fast growth lines are sometimes found with variations in protoplast yield. However, DAPI stainability shows significant positive correlation with protoplast yield (Table 2). High DAPI stainability lines tend to have higher protoplast yield. The viability of isolated protoplasts is generally above 85%. Variation in protoplast size (Fig. 1G) was frequently noted and the range varied from 8 μm to 28 μm in diameter with an average of about 18 μm. Sieving selection of suspension cells can not guarantee the high yield of protoplast. We sometimes found that some sieved cell clumps even had low DAPI stainability and the proto-

Table 2. *Summary of DAPI classification, average protoplast yield and plant regeneration frequencies in suspension cell lines derived from rice anther culture*

Cell lines[a]	DAPI[b] classes	Avg. protoplast yield (No/g calli)	Avg. regeneration frequency (%)
HC56 WL	L	5.3×10^4	28.4
HC56 WM	M	2.3×10^5	30.0
HC56 WH	H	3.0×10^7	40.1
HC56 SL	L	Trace	–
HC56 SM	M	1.2×10^6	25.0
HC56 SH	H	1.9×10^7	60.0
TP309 SL	L	5.0×10^5	26.7
TP309 SM	M	1.4×10^6	37.9
TP309 SH	H	7.8×10^6	38.4

[a] HC56 WL, WM, WH: cultivar Hsin-Chu 56, suspension initiated since winter 1988; HC56 SL, SM, SH: cultivar Hsin-Chu 56, suspension initiated since summer 1989; TP309 SL, SM, SH: cultivar Taipei 309, suspension initiated since summer 1989.

[b] Suspension lines with low, medium and high DAPI stainability. L: Percentage of suspension cells with DAPI stainability less or equal to 25%; M: Percentage of suspension cells with DAPI stainability between 25% and 70%; H: Percentage of suspension cells with DAPI stainability greater or equal to 70%.

plast yield was generally low. Between the two cultivars, HC56 suspensions tend to have small and fragile embryogenic cell clusters, while cells of TP309 tend to aggregate easily. The average protoplast yield is generally higher from HC56 than TP309. Although the average regeneration ability seems to increase with DAPI stainability, we find nuclear size and distribution pattern are also important factors for deciding regeneration ability. Cell lines with large nucleus, weak fluorescence, less compact or/and loose dispersion tend to have lower regeneration ability. Vigorous cell lines have brighter DAPI flourescence than less vigorous lines. Comparison between suspension cell lines from visible selection for fast growth lines and from selection for high DAPI stainability and nucleus pattern on protoplast plating efficiency as well as regeneration ability were studied. As shown in Table 3, plating efficiency and regeneration ability significantly increase with DAPI selection when proper cell lines were used as nurse cells. Nevertheless, line to line variations on protoplast division and cell colony development occurred frequently. Failure to have protoplast division and colony development were also observed when selection for DAPI stainability without noting the nuclear patterns and cell types. Poor DAPI stainability lines tend to have lower percentage of protoplast division, and not every high DAPI stainability line can develop into protoplast colonies. We find low DAPI stainability can change to high DAPI stainability with intensive care and proper subculture. However, as the subculture passage prolonged, the average plant regeneration declined. Selection for high DAPI stainability without noticing of nuclear size variations and cell patterns tends to lower the regeneration ability, too.

Table 3. *Protoplast plating efficiency and regeneration ability between selection and non-selection for high DAPI stainability*

Cell lines	DAPI selection	Plating[a] efficiency (%)	Regeneration frequency (%)
Hsin-Chu 56	No	5.2	20.0
Hsin-Chu 56	Yes	11.1	42.6
Taipei 309	No	0.4	–
Taipei 309	Yes	9.7	40.0

[a] Plating efficiency = (No. of colonies with at least 7-8 cells aggregate)/ (No. of cells/plated).

Discussion

Haploid protoplasts can be obtained from suspension lines derived from anther culture. This study indicates that after 3-4 months of subculture 50-60% of suspension cells remain haploid, however, prolonged culture significantly increase the percentage of multinucleate cells. Chen and Chen (1980) reported chromosome number change in microspore callus of rice during successive subcultures. Nishibayashi *et al.* (1989) also noted the chromosome variations among protoplast-derived calli and plants regenerated from the calli of rice. Spontaneous chromosome doubling through endomitosis or endoreplication are inevitable during the culture (Sunderland, 1977; Chen and Chen, 1980; Kanda *et al.*, 1988; Nishibayashi *et al.*, 1989). Therefore it should not be surprised that nucleus size variation is frequently observed from the DAPI staining.

Genotype differences in response to culture were also noted in this study. TP309 has been reported to succeed in plant protoplast culture and regeneration in other labortories (Albdullah *et al.*, 1986; Li and Murai, 1990). In this study, HC56 had a better growth rate in AA medium in comparison with TP309. Under our cultural conditions, TP309 had lower yield of protoplast than HC56. This is because TP309 suspension lines tend to aggregate more easily than HC56 in our culture. Nevertheless, when fine suspension lines were collected without passing through the steel mesh, we could obtain reasonable amount of protoplast for manipulation, too. Differences on the regeneration frequencies of suspension lines and protoplast-derived calli between two cultivars are not significant.

From this study, high DAPI stainability cell line tends to have high protoplast yield. However, the relative regeneration ability and plating efficiency are not completely relied on DAPI stainability. The relative cell type, nuclear size and nurse cell lines also play some roles in plant regeneration and protoplast division. Therefore, selection is not solely based on high DAPI stainability. The reason why high DAPI stainability lines tend to have high protoplast yield can be referred to either (1) actively dividing cells tend to have thin cell wall which permits the DAPI penetration more easily or (2) the non-stainable cells or slow DAPI -taken cells are actually aged or even nonvital.

Although DAPI was previously described by Coleman and Goff (1985) as a vital stain, some contrary evidence had been reported (Coleman *et al.*, 1989). However, our evidence from the FDA stainability, which was used as a vital stain for culture cells (Widholm, 1972), shows that low DAPI stainability cell lines tend to have low FDA stainability, too. We also try to isolate DNA from two cell lines, one with high DAPI stainability and the other with poor DAPI stainability. Results indicate that DNA can be isolated from the high DAPI stainability line but not from the poor stained line. This might support our suggestion that the DAPI non-stained cells were actually non-vital cells.

We also find that low DAPI stainability lines can change to high DAPI stainability, if medium refresh and/or proper subcultures are done. Nevertheless, even proper subculture is maintained, the decline in vigor of cell lines is still inevitable. The average regeneration ability is going down as subculture passages prolonged. However, the oldest suspension cell lines, which we maintained for about two years, still keep about 20% regeneration ability without selection for callus types. Fine dispersed cell clumps sieved through mesh, sometimes, can not obtain high protoplast yields. Through the DAPI stainability test, it is found that some small cell clumps even have poor DAPI stainability. Protoplast yield, plating efficiency and regeneration frequency can be improved via the aid of DAPI stainability selection (Table 2 and 3). Medium size of nucleus and more compact cell type (Fig. 1E) generally are indications of high plating efficiency of cell clumps. Cell type with large or small dispersed nucleus (Fig. 1F) generally yields large vacuole protoplasts and will not develop into cell colonies.

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水稻細胞懸浮系之 DAPI 螢光染色性選拔與原生質體分離及再生之探討

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細胞懸浮培養與原生質體純化量有極密切之關係。從水稻花藥來源細胞懸浮系之維繫培養上發現，即使已繼代培養了3-4個月以上，大部分懸浮細胞系之生長速率仍不甚穩定。利用 DNA 專一性螢光核染色劑 DAPI(4',6-diamidino-2-phenylindole)探討水稻花藥來源癒合組織之懸浮細胞系，在培養期間其細胞螢光核染色之分佈與變化情形，發現不同懸浮系之細胞，在培養期間對 DAPI 之染色性有不同程度之差異。依 DAPI 螢光染色之高低，可粗略將懸浮細胞分成高、中及低三類，此三類懸浮細胞之植株再生及原生質體純化量與螢光染色之高低有明顯之相關；一般 DAPI 染色率高之懸浮細胞系(≧70%)，其植株再生及原生質體之純化量均較其他二系爲高。高 DAPI 染色系之平均原生質體收穫量，新竹 56 號爲 2.5×10^7 個原生質體每克鮮懸浮細胞，而台北 309 號爲 7.8×10^6 個原生質體每克鮮懸浮細胞，亦偶有發現高達 10^8個原生質體每克鮮懸浮細胞者。以 DAPI 螢光染色法選拔染色率高之懸浮細胞系，且輔以核大小，分佈情形爲參考時，原生質體之平埋培養效率有明顯提高之趨勢。一些無法染色之細胞，以 FDA(fluorescein diacetate)測定其活性，證明確爲老化或無活性之細胞。