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Partial desiccation of mature embryo-derived calli, a simple treatment that dramatically enhances the regeneration ability of indica rice

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Abstract. Regeneration of indica rice varieties remains a limiting factor for researchers undertaking rice transformation experiments. As reported for japonica rice and other crops, partial desiccation of indica rice calli dramatically promotes organogenesis and leads to high regeneration ability. We are now able to obtain 66.5%, 61.1% and 73.7% of calli that regenerate plants for the indica varieties TN1, IR72 and IR64 whereas in non desiccated controls only 30.0%, 15.5% and 18.7% of calli regenerated, respectively. Plants obtained were phenotypically normal and 50% were highly fertile. Partial desiccation is a reliable and simple method for improving indica rice regeneration. It also shortens the time of in vitro culture.

Abbreviations: 2,4-D: 2,4-Dichlorophenoxyacetic acid; BAP: 6-Benzyl amino purine; DTT: Dithiothreitol; EDTA: Ethylene diamine tetra-acetic acid; MS: Murashige and Skoog; NAA: Naphtalene acetic acid; PAGE: Polyacrylamide gel electrophoresis; PAR: Photosynthetic active radiation, SDS: Sodium dodecil sulfate.

Introduction

Low yields caused by virus infections of economically important crops is one of the many problems that biotechnology is expected to address in part through plant transformation with resistance genes. In the case of rice, a large number of laboratories all over the world have been trying to develop transformation procedures. In most, if not all cases, the limiting factors for obtaining transformed plants lies in the regeneration of plant material rather than the transformation procedure itself.

Many laboratories are able to obtain high regeneration frequencies in japonica rice (Abdullah *et al.* 1986; Kyozuka *et al.* 1987; Li and Murai 1990; Wu and Zapata 1992). Nevertheless, many breeding programs and rice producing areas rely on indica rice varieties that provide the major source of calories for a large part of the world's population.

A few research groups have reported regeneration of Class I indica varieties (Kyozuka *et al.* 1988; Hartke and Lörz 1989; Lee *et al.* 1989; Datta *et al.* 1992). The number of regenerated plants in all cases is rather small (Kyozuka *et al.* 1988), and low fertility is often a problem (Datta *et al.* 1992). Despite the fact that these reports were followed by successful transformation experiments, the present technology is incompatible with transformation of large number of plants and requires technically advanced training and equipment. In order to be able to develop large scale transformation procedures in indica rice, regeneration frequencies must be improved, the methods simplified and the regenerated plants have to be highly fertile.

Partial desiccation has been reported to enhance somatic embryo differentiation and development in soybean (Hammatt and Davey 1987), grape vines (Gray 1987), wheat (Carman 1988), spruce (Roberts *et al.* 1991), and cassava (Mathews *et al.* 1993). Tsukahara and Hirosawa (1992) reported that this treatment was effective on japonica rice calli induced from cell suspension cultures.

In the present paper, we describe a simple method to enhance the regeneration frequency from mature embryo-derived calli and subcultured calli of three indica rice breeding lines: TN1, IR72 and IR64. We also show that regeneration is faster after partial desiccation and that most of the plants obtained are highly fertile.

Materials and Methods

Plant materials Rice seeds (*Oryza sativa* L., indica varieties TN1, IR72, and IR64) were provided by the International Rice Research Institute (IRRI, Los Baños, The Philippines) and are considered to be pure "Class I" indica lines according to Glaszmann (1987). Plants were propagated under standard greenhouse conditions, and were self-pollinated. Seeds were harvested and dried for 3 weeks in an oven at 30°C, and then stored in a dry and well aerated place at room temperature. These mature seeds were used as initial plant material for all the experiments.

Callus induction Mature rice seeds were gently dehusked in a mortar with a pestle, and surface sterilized by soaking in 75% ethanol for 3 min. with rotary agitation (75 rpm), and then in 1.3% sodium hypochlorite with 0.02% Tween 20 for 45 min. After three rinses in sterile deionized water, the seeds were incubated (15 seeds per plate) for 4 days at 30°C in the dark on

callus induction NB medium. NB medium contains N6 inorganic salts (Chu *et al.* 1975), B5 micro elements and vitamins (Gamborg *et al.* 1968), casein hydrolysate (300 mg/l), proline (300 mg/l), glutamine (300 mg/l), sucrose (30 g/l), agarose (type I, Low EEO, Sigma) (4 g/l), supplemented by 2,4-D (2 mg/l), NAA (1 mg/l) and BAP (1 mg/l). NB medium was brought to pH 5.8 and autoclaved at 120°C for 20 min. The small calli originating from the scutellar zone were separated from the endosperm and the shoot axis. They were incubated again on callus induction medium in a growth chamber at 25°C in the dark for an additional 12 days before being used in experiments. Control calli were transferred directly to regeneration medium whereas assay calli were partially desiccated as described below.

Subcultured calli Primary calli produced on induction medium were transferred to fresh NB medium every 20 days and kept in the dark. At the end of each cycle, pieces of the mother calli were separated and transferred independently to new medium. After 4 to 6 such cycles the subcultured calli were used for control and partial desiccation experiments.

Partial desiccation treatment Samples of 15 to 20 calli were transferred to an empty sterile Petri dish containing two sterile disks of Whatmann 3MM paper, and weighed. The Petri dishes were sealed with Parafilm and stored in a growth chamber at 25°C in the dark, for sufficient time to obtain the desired extent of desiccation. The "percentage of desiccation" refers to the ratio between the weight of the calli after desiccation and the weight before desiccation, multiplied by 100.

Plantlet regeneration After treatment, the partially desiccated calli were transferred to RN regeneration medium. RN = NB supplemented by NAA at 1 mg/l and BAP at 3 mg/l, brought to pH 6.0. The calli were incubated in a growth chamber for 12 days (day and night constant temperature of 25°C; 16 h day / 8 h night; fluorescent illumination of 100-125 mM. m-2.s-1 PAR), after which they were transferred to 1/2 strength MS medium (Murashige and Skoog 1962), supplemented by NAA (1 mg/l) and BAP (1 mg/l). As soon as leaves and roots began to proliferate, the plantlets were moved to Magenta boxes containing hormone-free MS medium.

Phenotypic characterization of regenerated plants and plant fertility To check for fertility, 10% of the plantlets in magenta boxes were chosen at random, transferred to soil, placed in a greenhouse and grown to flowering, anthesis and seed set. Half of these plants were scored for phenotypic characteristics such as plant height, number of tillers and panicles, pollen viability, percentage of fertility and germination ability of the seeds.

Fertility percentage (or panicular fertility) corresponds to the number of full spikelets over the total number of spikelet insertions on the panicles. In this work, the percentage of fertility was assessed by counting the number of full spikelets out of 300 spikelets on three panicles from three different tillers.

The first generation of seeds was tested for its ability to germinate and develop to a complete plant. Twenty mature seeds harvested from the parental plants were dried 14 days in an oven at 30°C, sterilized 8 min. in a 2% sodium hypochlorite solution and laid on several layers of sterile paper soaked with 0.1 M calcium chloride in Magenta boxes. The number of plantlets was counted after 14-16 days of germination and growth.

To determine pollen viability, one immature panicle was cut for each plant and fixed immediately in ethanol/acetic acid (3:1 v/v). Anthers were excised from 3 to 5 different flowers per panicle, laid down on a microscope slide and stained (Alexander 1969). Preparations were examined 5 min. after staining by light microscopy (Nikon Labophot, Japan). Viability percentage was determined using a minimum of 200 grains.

Polyacrylamide gel electrophoresis of callus protein One day after treatment, 0.2 mg of control or partially desiccated calli were removed from the regeneration media and frozen in liquid nitrogen. Calli were ground into powder with liquid nitrogen in a mortar. Powder was transferred to an Eppendorf tube and homogenized for 2 min. with 400 ml of protein extraction buffer 0.1 M Tris brought to pH 6.8 with HCl, 0.01 M EDTA, 5 mM DTT. After centrifuging for 10 min. at 10000 g, the supernatant was stored at -80°C. Concentrations of protein samples were estimated according to (Bradford 1976).

Gel analysis was performed in the modified Laemmli discontinuous buffer system (Laemmli 1970) using a 12% polyacrylamide analytical gel and a 5% polyacrylamide stacking gel each containing 0.1% SDS. High range molecular weigh markers (Gibco BRL) were used for calibration. Five µg of total protein were loaded per well after heating at 85°C for 5 min. Electrophoresis was performed for 1 h at 200 V / 85 mA. Gels were stained with Coomassie brilliant blue for 15 min. and subsequently destained (Sambrook *et al.* 1989).

Results

Callus induction and determination of the optimal desiccation level.

In our induction conditions we obtained calli 3 mm in diameter within 16 days. In order to prevent heterogeneity of water loss in the calli, all experiments were started with a pool of calli of similar size and shape. Seeds that did not germinate (between 1 and 2.5% according to the variety) were discarded as were calli that were obviously smaller than the average.

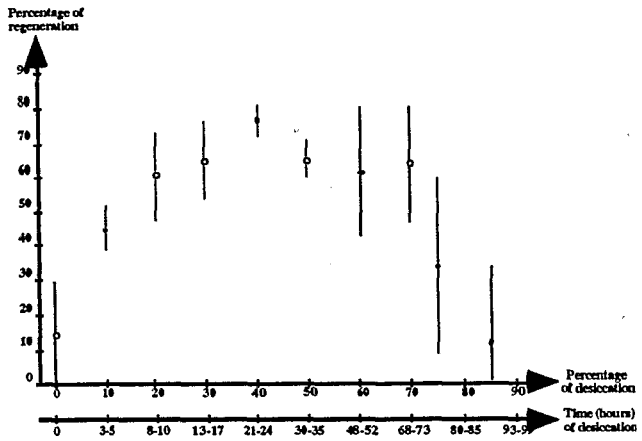

Figure 1: Effect of partial desiccation on the regeneration frequency of embryo derived calli. Experiment performed on TN1 variety. (Each point is the average of four independent experiments. Vertical bars represent means ± Standard Deviation).

In order to determine how the calli would respond to different levels of desiccation, four replicated kinetics of desiccation were undertaken on the cultivar TN1. The calli were dried sufficiently to lose 10, 20, 30, 40, 50, 60, 70, 75 or 85% of their fresh weight. They were then transferred to regeneration medium in the light to induce organogenesis. At the end of the regeneration process, the percentage of regenerated plantlets was determined. This percentage corresponds to the ratio of the number of regenerated plants (with shoots and a developed root system) to the number of calli treated, multiplied by 100. The results obtained are presented in Figure 1. The graph shows two distinct phases. In the first phase, from 0% to 40% of desiccation, the regeneration frequency increased continuously with the

percentage of desiccation. The calli responded quickly to partial desiccation. With 10% water loss regeneration tripled compared to the control and after 20% water loss regeneration increased to 61%. The maximum efficiency was obtained when calli lost 40% of fresh weight in all the replicates. In the second phase, from 40% to 85% of desiccation, the regeneration rate decreased with the level of water loss. Desiccation over 70% irreversibly damaged the calli and resulted in death around 85-90% of water loss.

Desiccation of calli induced from mature seeds.

Ten independent experiments were carried out with TN1 mature seeds, four with IR72 and two with IR64. In each experiment calli were desiccated to result in a loss of 30% - 50% fresh weight and the percentages of regeneration were then determined. Table 1 summarizes the results obtained over all the experiments for the partial desiccation treatments versus the controls. The highest percentages obtained during an experiment were the following: 86.3% of 113 calli for TN1, 74.4% of 43 calli for IR72 and 79.2% of 99 calli for IR64. Ratios of these values indicate that desiccation treatments provided an improvement of 2.2 fold for TN1, and 3.95 fold for both IR72 and IR64. Figure 2 shows the effect of desiccation on organogenesis and on plantlet regeneration.

Table 1. Effect of desiccation on the regeneration of plants from mature embryo derived calli and subcultured calli of the three class I indica varieties TN 1, IR 72, IR 64. (Calli were desiccated to 30-50%). ND: no desiccation, PD: partial desiccation, M E: mature embryo, Sub.: subcultured, Pl.: regenerated plants, %: percentage of regeneration

	TN 1		IR 72		IR 64	
	ND	PD	ND	PD	ND	PD
ME. Calli	566	1106	279	316	235	213
Pl.	170	726	43	193	44	157
%	30±15.5	66.5±11	15.5±18	61±17	19±3	74±7
Sub. calli	158	155	134	131	249	251
Pl.(albinos)	30(3)	107(10)	26(4)	78(12)	57(9)	136(18)
%	19±11	69±8	19±13	59.5±17	23±1	54±10.5

In addition to its positive effect on the number of calli regenerated, partial desiccation accelerated organogenesis and development and thus shortened the time in tissue culture. The first leaflets were obtained as early as 6 days after desiccation whereas for control calli the first leaflets appeared 9-10 days after transfer to regeneration medium. Plantlets with more than 3 leaflets and a developed root system were obtained 16-18 days post desiccation compared to 23-25 days for the control calli. The first plants were transferred from Magenta boxes to soil 32-34 days after desiccation whereas this took 45-50 days for control plants.

Fertility.

Phenotypic observations were recorded for plants that were transferred to the greenhouse and grown to maturity (i.e., 132 of the plants regenerated via in vitro culture). A large majority of the plants had no phenotypic changes compared to control plants grown directly from mature seeds. 6% (8/132) of the plants were smaller compared to the control plants, and 11% (14/132) of the plants had a lower number of panicles than the controls under the same

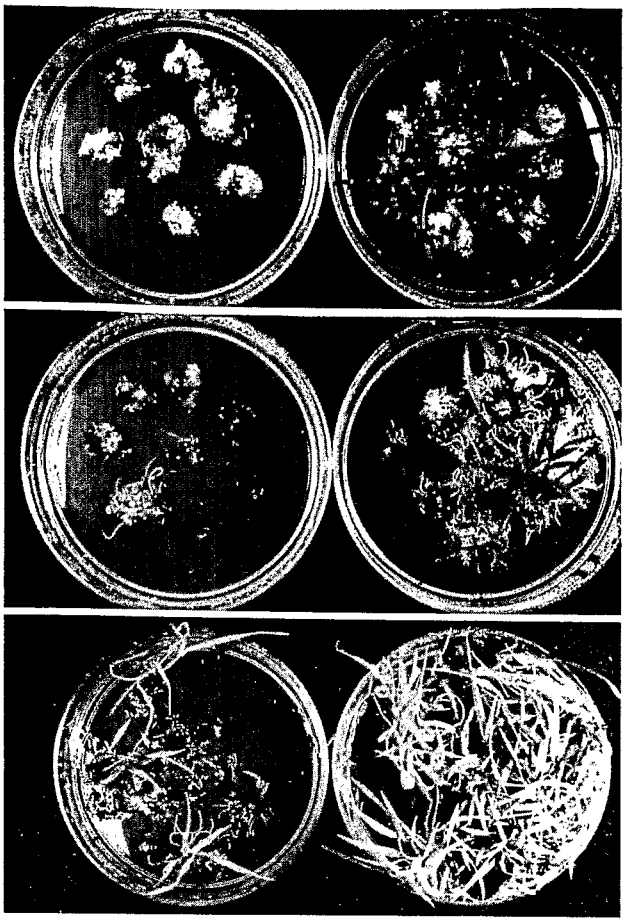

Figure 2: Effect of desiccation on indica rice regeneration for the three varieties, left plate shows control calli, without desiccation, right plate shows regenerating plantlets from partially desiccated calli. A: TN1, B: IR72, C: IR 64. (partial desiccation was 40% for TN1, 45% for IR72 and 64. Pictures were taken 25 days after treatment for A&B, 35 days after treatment for C.

growing conditions. No albino plants were obtained in any of the experiments with mature embryo derived calli. 83% of the plants bore a normal number of seeds and only 4% of the plants were completely sterile (Table 2). Starting from the transfer of the plants to soil in the greenhouse, flowering and seed set did not require a longer time for regenerated plants than for plants obtained from seeds. There was no correlation between a low panicular fertility and the ability of the seeds produced to germinate since all the seeds from the first generation of regenerated plants were able to germinate. Pollen viability was determined by differential staining. Viable pollen grains showed an intense pink staining and cytoplasm was visible, while dead grains remained empty and pale. Pollen viability of the regenerated plants was very heterogeneous, ranging from 0% to 100%. The mean value over all the plants assessed was 70.5% ± 23.6% (see Table 2). Determination of pollen viability of control plants grown in the same conditions showed less heterogeneity (ranging from 45% to 100%) with a mean value of 79.8% ± 19.3%. No differences were noticed between the different indica varieties and no correlation was found between high or total panicular sterility and a reduction in pollen viability.

Effect of desiccation on subcultured calli.

Effect of partial desiccation on plantlet regenerationfrom subcultured calli was also investigated. Calli obtained after 4 to 6 subcultures were used for treatments as if they were mature embryo-derived calli. Percentages of regeneration obtained are reported in Table 1. Frequencies of regeneration were slightly lower than for mature embryo derived calli but the improved regeneration after desiccation was evident. We obtained a 3.6 fold increase for TN1, 3.1 fold for IR72 and 2.4 fold for IR64. The average percentage of albinos was 12.9% overall, ranging from 9.3 to 16.0%, depending upon the variety and the treatment. No significant differences in percentage of albino plants were found between partially desiccated and control calli.

Table 2. Fertility data recorded on plants regenerated after desiccation treatment of mature embryo derived calli.

	80-100 % Highly fertile	40-80% Poorly fertile	<40% Highly sterile	0% Sterile	Average
Panicular fertility	50%	33%	13%	4%	71±24
Pollen viability	32.2%	60.8%	3.5%	3.5%	66±30

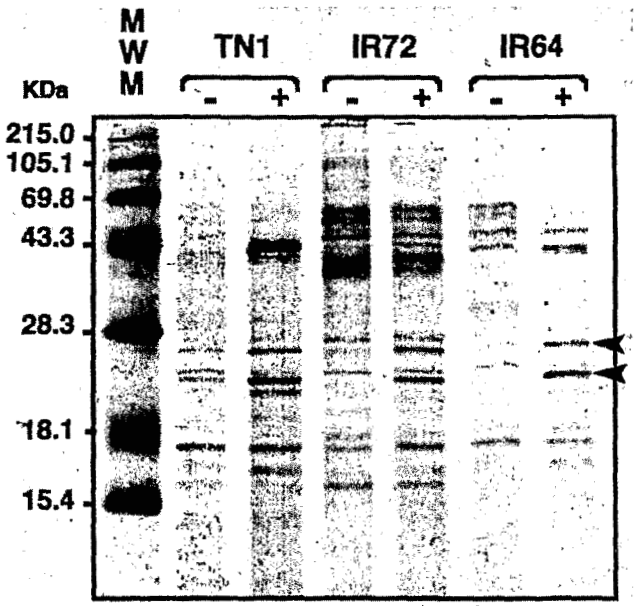

Figure 3: Comparison of soluble proteins from partially desiccated (+) and non desiccated (-) calli for the three varieties one day after treatment. Arrows point out the desiccation induced proteins of 22 and 26 kDa (lanes 3, 5, 7).

Protein content.

Total soluble proteins extracted from non desiccated or partially desiccated mature-embryo-derived calli were subjected to SDS-PAGE analysis and revealed a strong induction of two proteins one day after treatment (Figure 3). These proteins had molecular weights of 22 kDa and 26 kDa and were found in all three varieties after desiccation. These proteins were either not detected (IR72 and IR64) or were present in very low quantity (TN1) in the corresponding controls. When lanes are compared in pairs, additional induced proteins are apparent after desiccation, but none were found in all cultivars and/or not in each experiment.

Discussion

Japonica rice varieties usually show high regeneration abilities and behave very well through tissue culture process. Indica rice varieties, on the other hand, have poor regeneration potential. In the present study we succeeded in obtaining high regeneration frequencies of mature fertile plants from three Class I indica varieties. These high frequencies were obtained after partial desiccation of mature embryo-derived calli.

Callus induction was very efficient in terms of frequency, duration and quality. When the induction conditions were 30°C for 4 days, calli of suitable size were obtained in 16 days. Calli directly incubated at 25°C reached the same size 4-5 days later. Incubation at 30°C had no positive effect on regeneration frequency (data not presented). By selecting calli of similar size and shape partial drying was as uniform as possible in all the calli.

As reported by Tian *et al.* (1993), addition of cytokinins in both induction and regeneration medium also greatly contributed to induction efficiency. Lee *et al.* (1989), showed that addition of BAP in the regeneration medium had a positive effect on regeneration frequencies and number of plant produced. Such an induction protocol resulted in an average regeneration of 30.0% for controls TN1 calli.

Partial desiccation increased the regeneration of indica rice 2-4 fold. The best regeneration frequencies were obtained for desiccation treatments of 40%. This desiccation rate corresponded 21-23 hours of treatment. The frequencies obtained are comparable to those obtained for japonica rice varieties. Tsukahara and Hirosawa (1992) reported that dehydration of suspension culture-derived calli of the japonica variety "sasanishiki" enhanced their regeneration frequency from 5% up to 47%. Comparison of their experiments with ours shows several differences in both the methodologies and the results. 1) In our study, the intensity of the desiccation treatment has been expressed in terms of percentage of water loss rather than in terms of duration of the treatment in order to avoid the influence of ambient environmental factors, such as temperature, humidity and seasonal effects. 2) Their optimal regeneration frequency was obtained at 50% water loss, corresponding to 48 h. of treatment. In our case, the optimal regeneration frequency was reached at 40% and 22 hours. 3) Their effective range was narrow at around 50% desiccation whereas ours ranged from 25% to 75%. These major differences can be explained by differences in starting material.

A second interesting result of our study is the short period of in vitro culture of plant material. Starting from mature seeds, we were able to transfer the first plantlets from Magenta boxes to soil at day 48-50. Hartke and Lörz (1989) reported regeneration from mature embryos of the indica rice variety IR39385, but their regeneration protocol required 84 to 118 days before the plants could be planted in soil. Also, our short in vitro culture time leads to very few changes in the phenotypic and fertility characteristics of the regenerated plants. Half of our plants were highly

fertile with a panicular fertility over 80% compared to 85-95% for the control plants grown from seeds. We also showed that the desiccation procedure described did not lead to any particular development of male (pollen) or female fertility.

We also investigated the effect of partial desiccation on another type of starting material. Subcultured calli responded positively to the treatment although the regeneration frequencies were slightly lower. We also obtained a significant number of albino plants in both the control and desiccated pools of calli. Albino formation is therefore due to subculture conditions rather than to the desiccation treatment. The absence of albino plants when regeneration was from mature embryo derived calli reinforces this conclusion.

Kermode *et al.* (1989) reported that desiccation during seed development induced changes in translatable mRNAs and elicited modifications in protein synthesis. In our study, partial desiccation of mature embryo-derived calli led to differences in the soluble protein pattern as early as one day after the treatment. Desiccation triggers therefore rapid changes in development. We were not able to further characterize the induced proteins, or to determine if they were really involved in the developmental effects of desiccation.

Our study shows that it is now possible to obtain high frequencies of regeneration in indica rice. The method described is very simple, inexpensive and does not require advanced technical workers and equipment. Starting material is easy to obtain at any time during the year and does not require to maintain cell suspensions or subcultures. Moreover it seems to be cultivar independent, at least in indica varieties. Such a protocol can therefore be easily undertaken in a new laboratory or in countries where advanced laboratory facilities and technical staff are not available. Such regeneration frequencies should allow transformation procedures like particle bombardment to be more successful by giving rise to more transformed plants.

Acknowledgments

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References

Abdullah R, Cocking EC, Thompson JA (1986) Bio/Technology 4: 1087-1090.
Alexander MP (1969) Stain Technology 44(3): 117-123.
Bradford M (1976) Analytical Biochemistry 72: 248.
Carman JG (1988) PLanta 175: 417-424.
Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975). Scientia Sinica 18: 659-668.
Datta K, Potrykus I, Datta SK (1992) Plant Cell Reports 11: 229-233.
Gamborg OL, Miller RA, Ojima K (1968) Experimental Cell Research 50: 151-158.
Glaszmann JC (1987) Theoretical and Applied Genetics 74: 21-30.
Gray DJ (1987) Hortical Science 22: 810-814.
Hammatt N, Davey MR (1987) Journal of Plant Physiology 128: 219-226.
Hartke S, Lörz H (1989). Journal of genetics and breeding 43: 205-214.
Kermode AR, Oishi MY, Bewley JD (1989) In: Seed moisture pp 22-50, Stanwood and Mc Donald (Eds). CSSA special publication, Madison.
Kyozuka J, Hayashi Y, Shimamoto K (1987) Molecular and General Genetics 206: 408-413.
Kyozuka J, Otoo E, Shimamoto K (1988) Theoretical and Applied Genetics 76: 887-890.
Laemmli UK (1970) Nature 227: 680-685.
Lee L, Schroll RS, Grimes HD, Hodges TK (1989) Planta 178: 325-333.
Li Z, Murai N (1990) Plant Cell Reports 9: 216-220.
Mathews H, Schöpke C, Carcamo R, Chavarriaga P, Fauquet C, Beachy RN (1993) Plant Cell Reports 12: 328-333.
Murashige T, Skoog F (1962) Physiologia Plantarum 15: 473-497.
Roberts DR, Lazaroff WR, Webster FB (1991) Journal of Plant Physiology 138: 1-6.
Sambrook J, Fritsch EF, Maniatis T (1989). In: Molecular cloning, a laboratory manual, second edition. Cold spring harbor laboratory press, New York.
Tian W, Rancé IM, Sivamani E, Fauquet C, Beachy RN (1993) In vitro Cellular and Developmental Biology - Animal 29-A(3-II): 95-A. Abstract.
Tsukahara M, Hirosawa T (1992) Plant Cell Reports 11: 550-553.
Wu C, Zapata FJ (1992) Plant Science 86: 83-87.