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Evaluation of Total Protein Profiles Using 1-D and 2-D Gel Electrophoresis on Cotyledonary Node Explants Infected with Agrobacterium tumefaciens in Soybean [Glycine max (L.) Merr.]

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Abstract

The establishment of an efficient protocol for successful *in vitro* genetic transformation in soybean depends upon an effective interaction between cotyledonary node explants developed from germinated seeds and *Agrobacterium tumefaciens*. This study, therefore, evaluated the amenability of cotyledonary explants to *Agrobacterium* infection and co-cultivation, through total protein profiling using 2-dimensional gel electrophoresis.

Results showed that, germination of seeds on MS medium supplemented with 4.0 mgL^{-1} BA produced stout seedlings used to prepare competent explants. Protein lysates derived from these explants contained a combination of high and low molecular weight proteins, in which predominant polypeptides ranged between 10 to 120 kDa.

Thus, findings made showed varied protein profiles and intensity of protein spots detected on infected explants compared to the control, possibly as a result of explant co-cultivation with *Agrobacterium*.

Keywords: *Agrobacterium*, cotyledonary nodes, gel electrophoresis, protein profiles, soybean

INTRODUCTION

Soybean remains one of the most important leguminous grain crops cultivated in the tropics, subtropics and temperate regions mostly for commercial purposes. Globally, this is the number one oilseed crop widely grown for its rich oil, carbohydrates, essential mineral elements and protein content that are largely contained within the seeds [1]. The high amounts of essential amino acids and unsaturated fatty acids enables effective industrial processing into many consumable and health-promoting product ingredients. The concentrated and isolated forms of proteins contained in this crop have beneficially functional and nutritional applications in various kinds of agricultural, foods and pharmaceutical industries [2].

However, the sustainability of soybean production remain increasingly stalled by the frequently changing environmental conditions and the broader impacts of climate change. Reports clearly indicated that, environmental stress impedes biosynthesis and catabolism of metabolites yielding energy for activities that plants depend upon to sustain their life cycle. They cause physiological and phenotypic modifications such as wilting, chlorosis, necrosis, premature flowering, pod and flower abortions as an indication of metabolic stress [3–6]. Such effects exacerbate continued loss in yield, and are worsened by the recalcitrant nature of this crop to genetic manipulation. Genetic modification techniques such as *Agrobacterium*-mediated transformation, electro and chemical cell surface poration or direct protoplast-mediated DNA transfer are required to improve the agroeconomic traits of soybean. Limitations for the establishment of highly efficient transformation protocols such as; genotype specificity, lower frequencies and the lack of routinely applied transformation systems impact negatively on soybean genetic manipulation. A rapid and efficacious protocol for genetic transformation is a prerequisite, not only for yield trait improvement but also for gene function and molecular breeding studies of all legumes [7]. Furthermore, as *Agrobacterium tumefaciens*-mediated genetic transformation is the most preferred method of genetic transformation in soybean, especially for being rapid, cheap to perform and easily optimised by improving the infection and *in vitro* or *in vivo* regeneration efficiency, then it must be thoroughly explored.

Therefore, this study investigated the effect of *Agrobacterium*-explant interaction during co-cultivation, and evaluated the amenability of genotypes through total protein profiling of explants following co-cultivation with *Agrobacterium*. This was mainly carried out to compare the protein profiles of co-cultured explants with non-cultured explants which may lead to the identification of specific proteins that may promote or inhibit the efficient genetic transformation in soybean.

MATERIALS AND METHODS

Plant materials, germination and explant preparation

Soybean [*Glycine max* (L.) Merr.] seeds, cultivar Dundee, LS677, LS678, Peking, TGx1740-2F and TGx1835-10E were used. The seeds were surface disinfected using chlorine gas for 16-hours as described by Mangena *et al*. [8]. The decontaminated seeds were then inoculated for germination on Murashige and Skoog [9] basal culture medium supplemented with 4.0 mgL^{-1} benzyl adenine (BA) and incubated in a growth room for 10-days. After germination and seedling development , the 10-day old soybean seedlings were transversely cut on the hypocotyls (6–10 mm beneath the cotyledons) and their epicotyls excised-off at the cotyledonary junctions to obtain the double cotyledonary explants [8].

Bacterial culture and co-cultivation

The binary plasmid pTF101.1 with *Oryza cystatin -1* gene (*oc-1*) and phosphinothricin acetyl transferase *bar* gene for glufosinate-ammonium resistance transformed in *A. tumefaciens* strain EHA101 was used in this study. The bacterium was reinitiated and grown from a glycerol stock into a liquid yeast extract peptone (YEP) medium containing 50 mgL^{-1} kanamycin and 100 mgL^{-1} spectinomycin. Bacterial culture was incubated on an Orbital shaker (174 rpm) at 28°C overnight until the optical density reached 0.8–1.0. The culture was pelleted at 3500 rpm for 10-min and then resuspended in infection medium prepared as described by Paz *et al*. [10]. The double cotyledonary node explants prepared from aseptically excised seedlings were immersed in prepared *Agrobacterium* inoculum. Immersed explants were then incubated on a shaker (110 rpm) for 30-min, at room temperature. Double coty-nodes not infected with *Agrobacterium* were used as a control. Thereafter, 30 explants per Petri plate for each genotype were co-cultured with *Agrobacterium* on co-cultivation medium [10] overlaid with a sterile filter paper. The uninfected control explants were also co-cultured under similar conditions, and all co-cultivation plates were then incubated in a plant tissue culture growth room for 4-days.

Protein sample preparation, extraction and precipitation

After 4-days of co-cultivation, the co-cultured explants were briefly washed with sterile distilled water and homogenised into a fine powder in liquid nitrogen using a mortar and pestle. Homogenised tissues were then transferred into 15 mL sterile centrifuge tubes and kept at -80°C until use for protein extraction. Buffers used for protein extraction and fractionation were prepared using the different buffer components prepared according to the Bio-Rad Bulletin number 6040 [12]. For protein extraction and precipitation, a 1 mL of cold 10% (w/v) 2,2,2-trichloroacetic acid (TCA) and 0.07% (v/v) ß-mercaptoethanol acetone were added in 200 mg of ground cotyledonary node tissues. The mixture was vortexed for 1-min and incubated at -20°C for 2-hours. After incubation, the mixture was centrifuged at 10,000 x g for 5-min at 4°C to remove chlorophyll. The pellet was resuspended in 1 mL cold acetone containing 0.07% (v/v) ß-mercaptoethanol by vortexing and centrifuged at maximum speed for 20-min at 4°C. Pellet was then dried under vacuum for 2–5 min, resuspended in 1900 µL of lysis buffer and sonicated on ice for 3–6 rounds of 15-sec each at 20% power to solubilise the precipitated proteins. A 5 µL of 99% N,N dimethylacetamide (DMA) was added on the lysate and mixture incubated on a rotary shaker for 30-min at room temperature. A 2 M dithiothreitol (DTT) was added to quench excess DMA and the lysate centrifuged at 16,000 x g for 20-min at 4°C. The extracted protein supernatant were then transferred into sterile centrifuge tubes and kept at -80°C until use for gel electrophoresis.

Protein fractionation using 1-D and 2-D gel electrophoresis

For fractionation, 10 µL of extracted proteins were denatured by boiling for 5-min and loaded into gel wells after mixing with 10 µL of 4X concentrated sample loading buffer. Electrophoresis was carried out at 100 volts using a Bio-Rad power pack. After reaching the bottom of the resolving gel, fractionated gels were removed and placed into a staining solution. For 2-D gel electrophoresis, fractionated proteins were separated according to their charge using isoelectric focusing (IEF) on the IPG Runner cassette system at pH 3–10. IEF was performed using electric voltage of 175 to 2000 volts over 45-min and 2000 volts overnight. After the completion of IEF, the focused strips were run immediately on the sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein fractionation was conducted as described by Laemmli [11] and Bio-Rad Bulletin 6040 [12].

Visualisation of proteins, image acquisition and analysis

Coomassie Brilliant Blue R-250 was used for profiling of proteins in the gels, containing 0.1% (w/v) Coomassie Brilliant Blue R-250 in distilled water, 40% methanol and 10% glacial acetic acid. The gels were shaken using rotary shaker at room temperature and then de-stained using a mixture of 40% methanol and 10% glacial acetic acid. The gels were digitised by imaging with the scanner and the image analysed with the PD-Quest software using 8.1 software package. Protein spots were detected by the software based on the spots parameters chosen by the selection of the biggest, smallest and least intense spots. One master gel was selected to compare the differential identity of the expressed proteins.

RESULTS

This study compared protein profiles of the six selected soybean cultivars (Dundee, LS677, LS678, Peking, TGx1740-2F and TGx1835-10E) to evaluate protein expression patterns due to *Agrobacterium* infection and co-cultivation.

However, according to the results, germination of soybean seeds on MS medium containing BA proved to be beneficial for establishing stout seedlings required as an explant source. Variations in germination responses were recorded, with the highest mean germination percentage attained in LS677 (90%) and LS678 (85%) followed by Dundee (84%), TGx1835-10E (80%), TGx1740-2F (76%) and Peking (74%), respectively.

All genotypes gave high protein concentrations, except Peking which yielded slightly dilute protein lysate from TCA/acetone precipitation. High protein lysate obtained in Dundee, LS and TGx genotypes also indicated that, the extraction and precipitation by TCA/acetone method (Bio-Rad Bulletin 6040) was the best choice for the co-cultured cotyledonary tissues. There were no pellet loss and any signs of contamination in all protein lysates. This implied that, a wide array of physiochemical properties like size, charge and hydrophobicity of proteins did not interfere with the TCA/acetone precipitation method used.

Total protein profile of the six selected soybean genotypes were visualised on 1-D SDS-PAGE gels as shown in Fig. 1 (a) and (b). Results showed that, protein lysate contained a combination of low and high molecular weight proteins that migrated at approximately 3 to 100 kDa (Table 1). This was observed both on the control (Fig. 1 a) and *Agrobacterium* infected coty-node explants (Fig. 1 b). A 1-D analysis showed predominant polypeptides in the range between 20–99 kDa for Dundee, 12–120 kDa for both LS genotypes, 10–60 kDa in Peking and 12–100 kDa in TGx genotypes (Table 1). But, Peking produced lower concentration of proteins than any other soybean genotype used. Comparatively, most intense bands were produced by Dundee (S1) in contrast with Peking (S4).

Table 1. Protein range identified using one-dimensional gel electrophoresis in six selected soybean cultivars.

Soybean cultivar	Well number	Agrobacterium uninfected explants (kDa)	Agrobacterium infected explants (kDa)
Dundee	S1	20 – 99	10 – 100
LS 677	S2	12 – 100	10 – 120
LS 678	S3	12 – 120	5 – 120+
Peking	S4	10 – 60	10 – 100
TGx 1740-2F	S5	12 – 100	5 – 100+
TGx 1835-10E	S6	12 – 100	4 – 100+

Note: The molecular weight of proteins was estimated in kilodaltons (kDa) using E. coli unstained protein standard marker.

Furthermore, soybeans that are genetically linked produced similar trends of protein profiles as observed in both Fig. 1 a and b. For example, bands obtained in LS677 (S2) and LS678(S3), as well as bands found in TGx1740-2F(S5) and TGx1835-10E(S6) are somewhat similar. It was also determined through visual analysis that there were more protein bands on gels ran with lysate samples from the *Agrobacterium* infected double cotyledonary explants (Fig. 1 b) than in the control (Fig. 1 a). This indicated different levels of protein expressions, implying that other new proteins were probably expressed in infected explants, that were not appearing on uninfected coty-nodes.

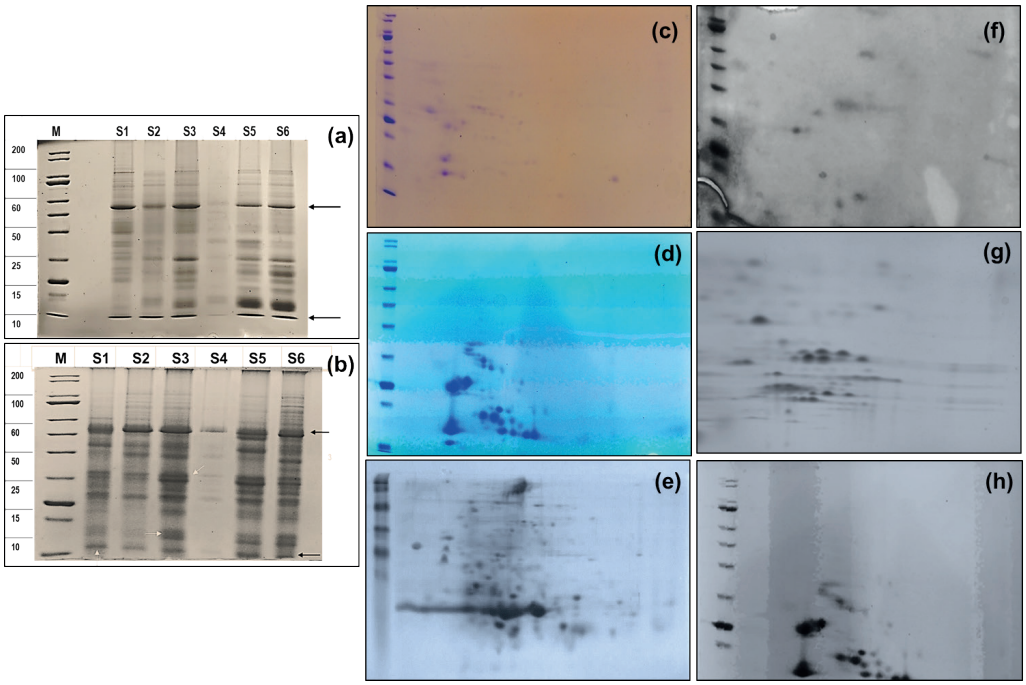

Fig. 1. Illustration of proteomic comparison between *Agrobacterium* co-cultured and uninfected cotyledonary node explants. (a) 1-D analysis of uninfected explants, (b) 1-D analysis of explants co-cultured with *Agrobacterium*, and examples of the total 2-D protein profiles for co-cultured explants in LS677 (d), Dundee (e), TGx1835-10E (g), LS678 (g) and uninfected explants in Peking (c) as well as TGx1740-2F (f).

As already indicated that the used bacterial culture density may have caused changes in the patterns of proteins expressed by explant tissue. By contrast, gels obtained form the control had different protein patterns compared to *Agrobacterium* infected extracts. More protein spots were identified from proteomic analysis of coty-nodes infected with *Agrobacterium*, especially for LS677, Dundee, TGx1835-10E and LS678 (Fig. 1 d, e, g and h). A less intense differential distribution was also observed on control gels for all genotypes used, with Peking and TGx1740-2F yielding a significantly lower number of spots among all the genotypes. Although the majority of these differentially displayed protein spots ranged between 5 and 80 kDa of approximated molecular weights, only LS677, LS678, Dundee and TGx1835-10E exhibited a clearly identifiable differences in the protein expression patterns observed, as exemplified in Fig. 1.

DISCUSSION

The positive responses in germination allowed for the successful preparation of sufficient and highly suitable cotyledonary explants used for the infection and interaction of explants with *Agrobacterium*. This indicated that, high seedlings vigour and well rejuvenated bacterial culture are a prerequisite for efficient co-cultivation of explants. The observation made was similar to previously made findings by Paz *et al*. [13] and Mangena *et al*. [14]. These reports also indicated that, the addition of plant growth regulators (e.g. BA) resulted in delayed necrotic and chlorotic symptoms observed on the co-cultured explants, which was reported to be mainly controlled by both bacterial infection and the duration of co-cultivation.

TCA/acetone precipitation gave high quality of protein lysates as recommended by Rajalingan *et al*. [15]. The report clearly showed inconsistencies using other alternative

methods, including the loss of pellet and less effectiveness of TCA alone in precipitating complex proteins, particularly for plant tissue samples. Observations made were in line with Natarajan *et al*. [16] and Xu *et al*. [17] emphasis on the simplicity of TCA/acetone protocol for precipitation of a dynamic range of proteins and reproducibility of the protein separation. Protein samples showed very minimal degradation and no contamination as indicated by Harder *et al*. [17]. The extraction protocol have undoubtedly and reproducibly trapped comprehensive repertoire of proteins without degradation or contamination.

The 1-D gel analysis also showed variably low polypeptide profiles for Peking compared to other genotypes. This could mean that the ratio of protein concentration to TCA/acetone precipitate was significantly low, further suggesting low protein yield by this homogenate. According to Islam *et al*. [19] this may be improved by additional grinding of sample material to improve solubility of the extracted proteins. However, Peking also had a lower mean germination rate, probably due to delayed imbibition or dormance as previously reported by Mangena and Mokwala [1], compared to all other genotypes. The results obtained during SDS-PAGE may also mean that, seed germination in Peking was directly proportional to the yielded protein concentrations of the explants. This may shed some light on the deterioration of seeds, immediately after harvest, and seed viability which remains momentary as indicated by the abovementioned report [1]. As genetically expected, similar genotypes presented relatively similar protein bands. Meanwhile uninfected explants of the same genotypes exhibited a few number of bands and protein spots than infected double coty-nodes. Intensified bands appeared between 25 and 40 kDa, with approximated molecular weights of about 26 kDa and 40 kDa. This is a possible influence of polypeptide response due to co-cultivation of explants with *A. tumefaciens* which may have induced some kind of physiological stress on the infected explants. Kirova *et al*. [20] reported varied protein expression due to physiological stress caused by drought in soybean cultivar Hodgson.

According to this report [20], the quantity of expressed proteins was evidently high under drought stress compared to the control well-watered soybean plants. Similarly, the explant infection and co-cultivation with *Agrobacterium* could cause physiological stress that may lead to production of additional stress related proteins or expression of transgene proteins. In case of this study, it could be the transcription of cystatin proteins encoded from the *oc-1* gene of the pTF101.1 vector used. The overall results obtained, including protein spots expressed, suggested that these proteins may have been expressed in response to co-cultivation of explants with *A. tumefaciens*. Such observations could be furthermore used to confirm possible transformation events during the genetic improvement of legume plants using *A. tumefaciens*-mediated genetic transformation. However, the low abundance and tricky identification of protein spots observed in genotypes such as Peking and TGx1740-2F may be due to the low abundance nature of proteins in the rapidly deteriorating seeds, poor explant vigour or the poor gel resolution generated through 2-D SDS-PAGE. Possible heavy posttranslational modifications [21] or limitations of isoelectric focussing may have as well contributed.

In conclusion, although the interaction between soybean genotypes and *A. tumefaciens* have been well studied at physiological and morphological level, no reports elucidated the alteration of protein profiles after co-cultivation. Thus, findings made showed varied protein profiles and intensity of protein spots detected on infected explants compared to uninfected double coty-nodes. Apart from the variations observed, 2-D SDS-PAGE could serve as an important tool to identify and analyse different genotypes, and for the selection of transgenic from non-transgenic soybean plants.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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