of protein sample [7]. OGE has also been successfully used to separate antibody charge isoforms prior to capillary IEF [8]. Initially devised for fractionation of proteins prior to gel-based techniques, OGE was soon employed to fractionate peptides prior to reverse phase (RP) liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) [9]. In such shotgun proteomics approaches, proteins can be enzymatically digested after [10] or before [11] OGE prefractionation; alternatively a sample can be fractionated twice, first using proteins and second using tryptic peptides [12]. Protein prefractionation using first OGE, second RP-LC, then followed by trypsin digestion and LC-MS/MS analysis was found to be a reproducible strategy to study complex samples [13]. The OGE device allowing unbiased fractionation of both proteins and peptides is now commercialised under the trade name of OFFGEL fractionator (Agilent Technologies) and was first reported by Hörth *et al*. [11]. A highly efficient quantitative strategy combines peptide labelling with isobaric tag for relative and absolute quantitation (iTRAQ) and OGE prefractionation prior to LC-MS/MS analysis [14-16]; it also enhances the identification of low abundant proteins [17]. Very recently, OGE was employed to explore phosphoproteomes [18, 19].

More publications, thus far limited to industrial or medical research, have made full use of this additional resolving step, mostly in a shotgun approach capacity, either fractionating proteins [20, 21], peptides [22-24] or both [25, 26]. However, to date, OGE has yet to be used on plant tissues. Our lab is interested in elucidating wheat responses to fungal pathogens; proteomics is one of the strategies adopted through the use of both gel-based and gel-free approaches. Here we report for the first time the OGE prefractionation of plant proteins. Soluble proteins were recovered from wheat whole leaves and separated using Agilent 3100 OFFGEL fractionator either using the purchased Agilent's kit or in-house solutions. The quality of protein prefractionation was assessed by 1-DE and 2-DE which helped comparing both the manufacturer's and our in-house protocols. Our in-house protocol was applied to apoplastic fluids from wheat leaves. Whether using whole leaf or apoplast samples, two-dimensional patterns were greatly enriched in spots following OGE prefractionation.

2. Materials and Methods

1. *Wheat culture and sampling*

Wheat (*Triticum aestivum* L. cv. Grandin) was grown from seeds. Prior to sowing, seeds were sterilised by incubation in 0.1% v/v sodium hydroxide/5% v/v ethanol for 10 min followed by three washes in sterile water. Ten sterilised seeds were sown per 2 L pot full of vermiculite grade 3 (Australian Perlite, Sydney NSW Australia) with 10 pellets of slow release Osmocote Exact fertiliser (Scotts Australia, Baulkham hills NSW Australia). Pots were placed in a sunlit greenhouse kept at 20°C and fully watered daily. All seeds had germinated after 4 days and the first leaf emerged from the cotyledon after 9 days. First true leaf leafs were collected after 12 days. Leaf samples for whole protein extraction were pooled into a tube and instantly frozen by immersion in liquid nitrogen and stored at -80°C until use. Leaf samples for apoplast recovery were used fresh, immediately after sampling.

2. *Apoplast recovery*

Apoplastic fluid was recovered according to Solomon and Oliver [27]. Briefly, leafs were cut into 3cm-long pieces and placed into a 35 mL syringe barrel along with 15 mL ddH_2O. By adapting the tip into a rubber stopper and operating the plunger, ddH_2O was forced into the leaf pieces through the stomata until the leaves turned dark green. Leaf pieces were retrieved using tweezers and the residual water on their surface was blotted with paper towels. The dry leaf pieces were transferred into a 10 mL syringe barrel without adjusting the plunger. A 1.5 mL Eppendorf tube was adapted at the end of the syringe tip and both the syringe and the Eppendorf were inserted into a 50 mL Falcon tube. The apoplastic fluids were recovered by centrifugation using a swing rotor for 5 min at 4000 x*g* at room temperature. Ten leaves produced approximately 0.5 mL of fluid. Apoplastic samples were stored at -80°C until use.

3. *Protein extraction from whole leaves*

Frozen leaf samples were finely ground in liquid nitrogen using a chilled mortar and pestle. Soluble proteins were extracted using a trichloroacetic acid (TCA)/ 2-mercaptoethanol (2-ME)/acetone method devised by Damerval *et al*. [28]. Briefly, the frozen powder was transferred into chilled 50 mL Nalgene Oak Ridge tubes (Thermo Fisher Scientific, Scoresby VIC Australia) and filled up with ice-cold 10% w/v TCA/0.007% v/v 2-ME/acetone solution. Following homogenisation, tubes were left to incubate at -20°C overnight. Tubes were centrifuged for 30 min at 12,000 x*g* and -10°C and the supernatant was discarded. Pellets were rinsed three times in ice-cold 0.007% v/v 2-ME/acetone solution and recovered by centrifugation (30 min, 12,000 x*g*, -10°C). Pellets were dried at room temperature overnight and solubilised in 1 mL resuspension (R) solution (7M urea, 2M thiourea, 4% w/v 3-[3-(cholamidopropyl) dimethylammonio]-1-propane-sulfonate, 1% w/v dithiothreitol, 1% v/v 2-ME, 10mM tris-(2-carboxyethyl)-phosphine-HCl, 0.5% ampholites 3-10). Protein extracts were transferred into 2 mL Eppendorf tubes and stored at -20°C until use.

4. *Protein content assay*

The protein content of both leaf protein extracts and apoplastic fluid samples was estimated using 2-D Quant Kit (GE Healthcare, Ryldamere NSW Australia) following the

Table 1. Consumables used for each OFFGEL fractionation method on wheat leaf protein samples.

consumable	Agilent method				in-house method			
	supplier	part number	grade purity	amount	supplier	part number	quality	amount
urea	Agilent	5188-6435	Electrophoresis grade	2 x 25.2 g	AMRESCO	0568-1KG	Ultra pure grade >99.9%	1 kg
thiourea	Agilent	5188-6436	ReagentPlus grade >99.9%	2 x 9.1 g	Sigma	T7875-100G	ReagentPlus grade >99%	100 g
DTT	Agilent	5188-6439	Electrophoresis grade	2 x 0.6 g	Sigma	D9779-5G	Molecular biology grade >99%	5 g
50% glycerol	Agilent	5188-6440	GC grade >99%	2 x 10 mL	Merck	4.10015.0500	GR grade >99.5%	500 mL
3-10 OFFGEL buffer	Agilent	5188-6437	n.a.	1 mL	GE Healthcare	17-6000-87	n.r.	1 mL
mineral oil	Agilent	5188-6443	n.a.	100 mL	Sigma	M5904-500ML	Molecular biology grade	500 mL
ddH_2O	Millipore	n.r.	MilliQ grade	n.r.	Millipore	n.r.	MilliQ grade	n.r.
24 cm 3-10 IPG strip	Agilent	5188-6442	n.r.	8 units	GE Healthcare	12-6002-44	n.r.	12 units
electrode pad	Agilent	5188-6434	n.r.	90 units	Bio-Rad	165-4071	n.r.	500 units
24 well frame	Agilent	G3100-44503	n.r.	6 units	Agilent	G3100-44503	n.r.	6 units
24 well frame lid	Agilent	G3100-47100	n.r.	6 units	Agilent	G3100-47100	n.r.	6 units
tray	Agilent	G3100-60007	n.r.	2 units	Agilent	G3100-60007	n.r.	2 units
electrode	Agilent	G3100-60002	n.r.	2 sets	Agilent	G3100-60002	n.r.	2 sets

n.a. not available, n.r. not relevant

manufacturer's instructions.

5. *OFFGEL fractionation*

Proteins were fractionated into 24 fractions by liquid isoelectric focusing (IEF) using the Agilent 3100 OFFGEL fractionator (Agilent Technologies, Forest Hill VIC Australia). Two methods were tested for OFFGEL fractionation of whole leaf samples. 1/ In the Agilent's method, fractionation was performed using the Starter kit (Agilent Technologies Part No. 5188-6444) which includes all necessary consumables. 2/ In our in-house method, fractionation was performed by, whenever possible, replacing all the consumables provided by Agilent's starter kit with their lab equivalents listed in Table 1. The 1.25X OFFGEL stock solution (50 mL) for high resolution separation along 3-10 pH range was prepared following the manufacturer's instructions, aliquoted into 2 mL Eppendorf tubes and stored at -20°C until use.

Parts were assembled, Immobilised pH gradient (IPG) strips were rehydrated, and protein samples were diluted into the 1.25X OFFGEL stock solution as instructed in the Agilent Quick Start Guide. The same wheat leaf sample was separated on two different IPG strips placed in the same tray following the Agilent or the in-house method. A total of 0.5 mg of leaf proteins were loaded onto each IPG strip. The apoplast sample was fractionated during a separated experiment following our in-house method only, by loading 1 mg of apoplastic proteins.

Both OFFGEL fractionation runs for leaf and apoplast samples respectively were performed using the preset program OG24PR00 (64kVhrs, 8000V, 50µA, 200mW). At the end of the runs, fractions were transferred into individual 1.5 mL Eppendorf tubes. Collected fractions were stored at -20°C until further use.

6. *pH measurement of the OFFGEL fractions*

Fraction pH values were estimated by diluting 2µL into 20µL ddH2O and wetting 0-14 pH indicator strips (Merck, Kilsyth VIC Australia). Values are displayed in Table 2 and illustrated in Figure 1. The expected pH values *per* fraction were calculated according to the IPG strip supplier data (24 cm 3-10 pH) and the OFFGEL well dimensions (24 x 1 cm long wells). The 7 pH units along 3-10 range were divided by 24 to give a value of approximately 0.3 pH units for the range covered by each fraction.

7. *One-dimensional electrophoresis (1-DE)*

Two micrograms of unfractionated protein samples or 10 µL of each fraction were diluted into up to 20 µL of Laemmli Sample Buffer (Bio-Rad, Gladesville, NSW, Australia). Broad Range SDS-PAGE Molecular Weight (MW) Standards (Bio-Rad) were used for MW reference. Samples were boiled for 5 min and loaded onto 4% homecast stacking polyacrylamide gels on top of 12% homecast resolving polyacrylamide gels

Table 2. pH values of OFFGEL fractions. Difference with expected value is indicated in bracket.

Fraction	Expected pH	Observed pH	
		lamina/Agilent method	lamina/in-house method
1	3.00	3.00 (+0.00)	3.00 (+0.00)
2	3.30	4.00 (+0.70)	4.00 (+0.70)
3	3.60	4.25 (+0.65)	4.25 (+0.65)
4	3.90	4.50 (+0.60)	4.50 (+0.60)
5	4.20	4.75 (+0.55)	4.75 (+0.55)
6	4.50	5.00 (+0.50)	5.00 (+0.50)
7	4.80	5.12 (+0.32)	5.15 (+0.35)
8	5.10	5.25 (+0.15)	5.30 (+0.20)
9	5.40	5.37 (-0.03)	5.45 (+0.05)
10	5.70	5.50 (-0.20)	5.60 (-0.10)
11	6.00	5.62 (-0.38)	5.75 (-0.25)
12	6.30	5.75 (-0.55)	6.00 (-0.30)
13	6.60	5.87 (-0.73)	6.15 (-0.45)
14	6.90	6.10 (-0.80)	6.30 (-0.60)
15	7.20	6.25 (-0.95)	6.45 (-0.75)
16	7.50	6.40 (-1.10)	6.60 (-0.90)
17	7.80	6.55 (-1.25)	6.75 (-1.05)
18	8.10	6.70 (-1.40)	7.00 (-1.10)
19	8.40	6.85 (-1.55)	6.75 (-1.65)
20	8.70	7.00 (-1.70)	6.75 (-1.95)
21	9.00	7.30 (-1.70)	6.75 (-2.25)
22	9.30	7.60 (-1.70)	6.75 (-2.55)
23	9.60	8.00 (-1.60)	8.00 (-1.60)
24	9.90	8.50 (-1.40)	8.00 (-1.90)

(10 wells each, 0.75 x 10 x 8 cm). Runs were performed using a Mini-PROTEAN Tetra Cell (Bio-Rad) system at room temperature for 10 min at 40V followed by 60 min at 180V.

8. *Two-dimensional electrophoresis (2-DE)*

A total of 100 µg proteins from unfractionated samples were diluted into R solution to reach a volume of 470 µL and then loaded onto 24 cm 4-7 or 7-10 IPG Strips (Bio-Rad) through in-gel rehydration. Fractions were pooled so that their pH values was included into the pH ranges offered by Narrow Range 24 cm ReadyStrip IPG strips (Bio-Rad) as indicated in Table 3. Pooled fractions were diluted into R solution to reach a volume of 470 µL and were loaded onto IPG strips whose pH ranges overlapped theirs through in-gel rehydration. IPG-IEF was performed using IPGphor II (GE Healthcare) and the following program: 0V for 1h after which mineral oil was added, 50V for 12h, 200V for 30min, 500V for 30min, 1000V for 1h, 8000V for 90,000VHrs. IPG strips equilibration was performed according to Görg *et al.* [29] by incubation into 1% DTT for 15 min following by incubation in 2.5% iodoacetamide for 15 min. Equilibrated IPG strips were transferred onto 12% homecast polyacrylamide/ bisacrylamide (37.5:1) gels (0.1 x 24 x 20 cm) and sealed in 1% (w/v) agarose (Invitrogen, Mulgrave VIC Australia) in Laemmli [30] running buffer with 0.002 % (w/v) bromophenol blue (BPB). Second dimension was performed using the Ettan DALT six Electrophoresis Unit (GE Healthcare) at 10°C at 40V for 30min followed by 450V for 4h30 (0.4mA, 100W).

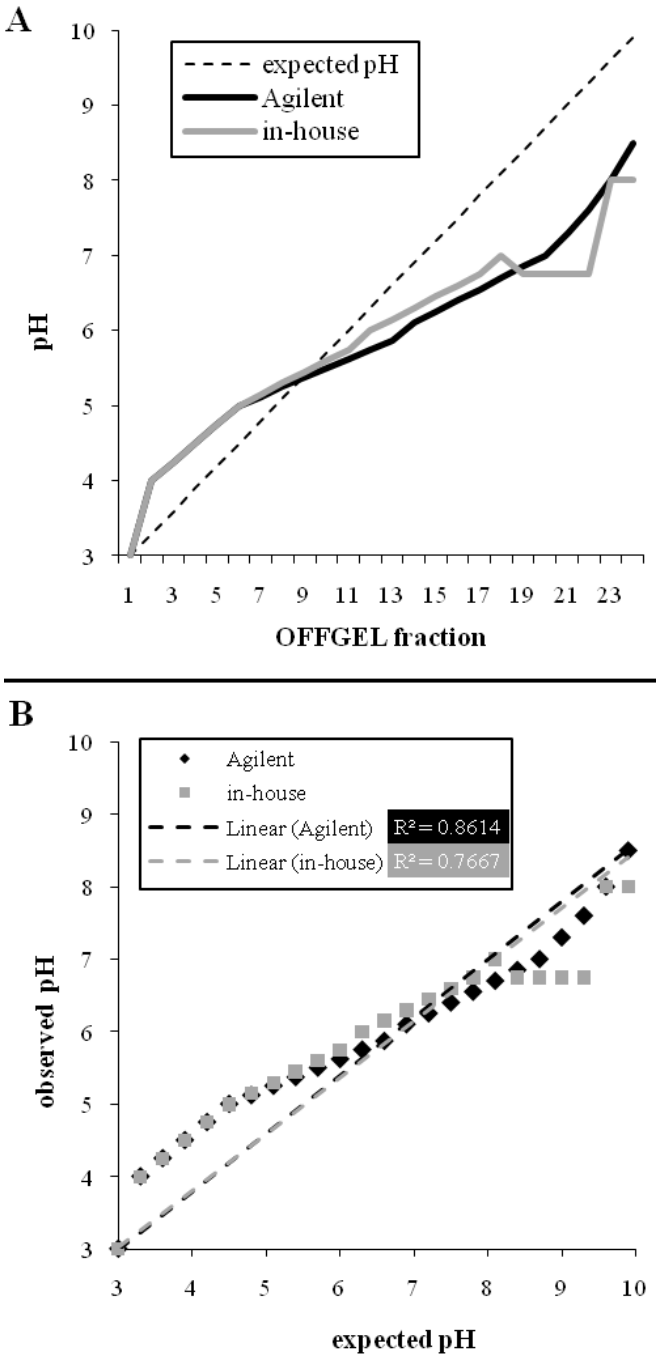

Figure 1. pH values of the OFFGEL fractions. A) Distributions of observed pH values. The dotted line represents the expected pH distribution. B) Scatterplots of the observed values of the fractions relative to the expected values. Dotted lines represent linear trendlines with intercept set to 3. Corresponding correlation rates (R^2) are indicated.

Table 3. Fraction pooling and IPG-IEF conditions

Sample	OFFGEL method	Fraction pooling	Fraction volume	pH range	Figure number
wheat leaf	Agilent	1 to 5	0.64 mL each	3-6	3A
wheat leaf	Agilent	6 to 11	0.54 mL each	4-7	3B
wheat leaf	Agilent	2 to 13	0.27 mL each	4-7	3C
wheat leaf	Agilent	12 to 17	0.54 mL each	5-8	3D
wheat leaf	Agilent	17 to 24	0.51 mL each	7-10	3E
wheat leaf	in-house	1 to 5	0.64 mL each	3-6	3F
wheat leaf	in-house	6 to 11	0.54 mL each	4-7	3G
wheat leaf	in-house	2 to 13	0.27 mL each	4-7	3H
wheat leaf	in-house	12 to 17	0.54 mL each	5-8	3I
wheat leaf	in-house	17 to 24	0.51 mL each	7-10	3J
wheat leaf	none	none	-	4-7	3K
wheat leaf	none	none	-	7-10	3L
wheat apoplast	in-house	1 to 10	0.32 mL each	3-6	4B
wheat apoplast	in-house	1 to 15	0.20 mL each	4-7	4C
wheat apoplast	in-house	8 to 17	0.32 mL each	5-8	4D
wheat apoplast	in-house	17 to 24	0.45 mL each	7-10	4E
wheat apoplast	none	none	-	4-7	4F
wheat apoplast	none	none		7-10	4G

9. *Gel staining*

Both one- and two-dimensional gels were stained following the silver nitrate staining method developed by Rabilloud and Charmont [31]. Stained gels were scanned using Molecular Imaging PharosFX Plus system (Bio-Rad) at 100 µm resolution with the densitometry mode. Images were converted into *TIF*-format (16-bit greyscale, 254 dots per inch).

3. Results and Discussion

A number of recent proteomics projects incorporating OGE attests to the ever-increasing interest in the use of this novel technology. Despite its growing acceptance, OGE has yet to be reported for plant systems. The first objective of this study was to trial OGE prefractionation of a complex plant protein sample by comparing the manufacturer's method with an in-house protocol, with the final aim of minimising consumable expenses. To this end, using the Agilent 3100 OFFGEL fractionator high resolution mode (24 fractions *per* 24 cm 3-10 IPG strip), two protocols were tested: 1/ the Agilent protocol based on the use of the Agilent's proprietary starter kit, and 2/ our in-house protocol for which all required solutions were prepared using electrophoretic grade consumables routinely used in our laboratory, IPG strips and electrode pads were purchased separately from another supplier. The difference in methods is described in Table 1. The second objective of this study was to protein-enrich two-dimensional patterns from two different plant tissues, whole leaf and leaf apoplastic fluids, using an initial protein prefractionation step, compared to two-dimensional patterns obtained from unfractionated samples. Both the Agilent protocol and our in-house method are assessed and compared using three approaches: pH distribution, one-dimensional profiles and two-dimensional patterns.

1. *The Agilent and in-house methods produce comparable electrophoretic patterns.*

Leaf samples were prefractionated into 24 fractions along a 3-10 pH range using OGE. Table 2 (graphically represented in Figure 1A) lists the pH values of each OGE fraction and indicates how different they are from the expected values, calculated by dividing the seven pH units of 3-10 gradient by the number of compartments (24). Considering leaf samples, the pH distribution obtained following the Agilent method skews less from the expected distribution than that obtained with our in-house method, differing at the most by 1.7 pH unit (fractions 20 to 22). A consistent trend appears across methods (Agilent and in-house) with the acidic fractions (up to pH 5.60) always displaying a pH value superior to the expected one, while the pH of basic fractions is constantly below the expected value. Basic fractions present the greatest pH difference with the expected distribution, as high as 2.55 pH unit (in-house method, fraction 22). The pH of the fractions distributes linearly as illustrated by the trendlines fitting the scatterplots of the expected pH values *versus* the observed ones (Figure 1B). The Agilent method (R^2=0.86) produces slightly more linearly distributed fraction pH from leaf sample than our in-house method (R^2=0.77). A linear distribution was also reported in *P. falciparum* system; pH values were mostly above the expected distribution, especially within very acidic fractions [20].

To further assess the methods' efficiency, each fraction was

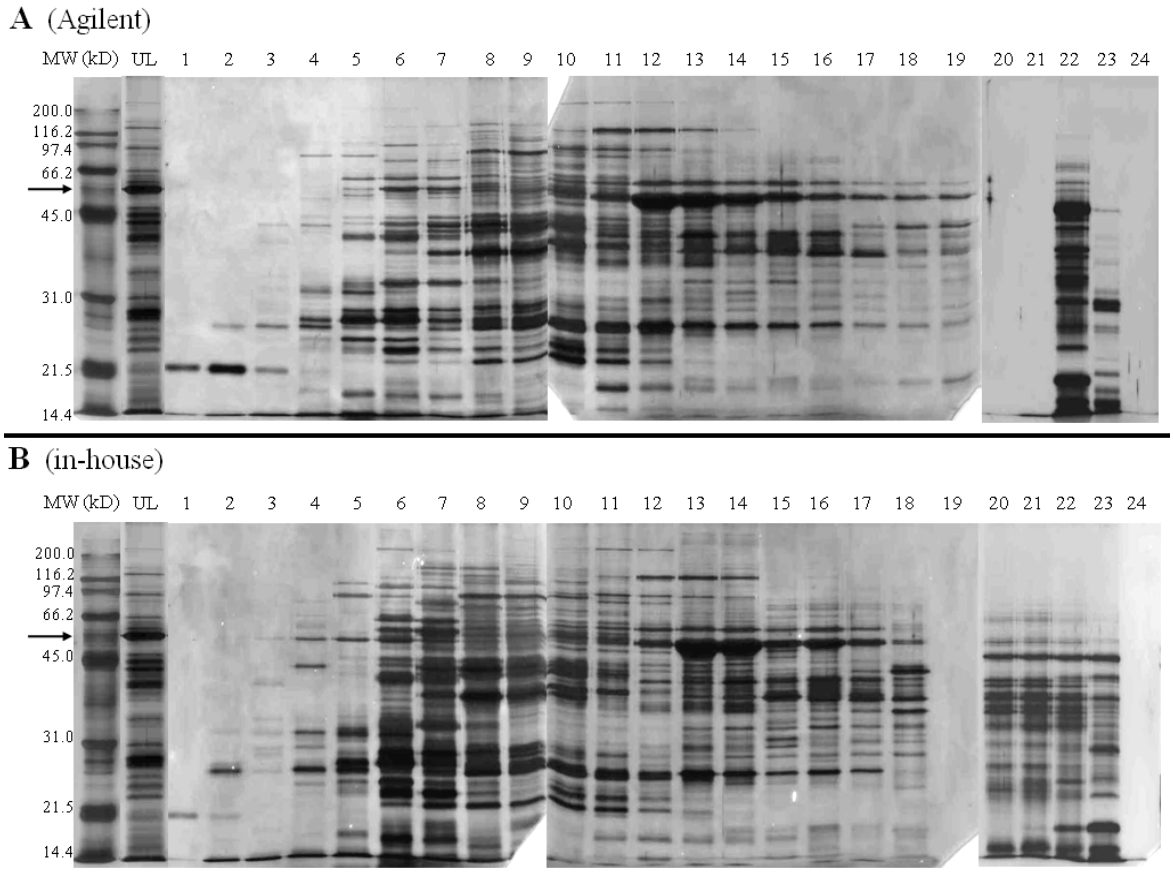

Figure 2. One-dimensional profiles of the OFFGEL fractions relative to those of unfractionated samples. A) Leaf sample fractionated according to Agilent's method. B) Leaf sample fractionated according to our in-house method. MW, molecular weight standards; UL, unfractionated leaf sample; 1 to 24, OFFGEL fractions 1 to 24. Arrows indicate the large subunit of RuBisCO.

separated using 1-DE, along with the initial unfractionated sample used as a reference (Figure 2). Comparing one-dimensional profiles of fractions obtained following the Agilent method to those obtained using our in-house protocol, it was evident that both methods produce excellent electrophoretic patterns, displaying well-resolved bands even within the most complex fractions. Proteins bands that are faint in unfractionated leaf samples (lanes UL) become much more prominent in some of the fractions, especially for proteins of very high or very low MW. In both methods (Figure 2A and B), very acidic fractions (1 to 5) are depleted in proteins, whereas acidic to neutral fractions (6 to 16) contain most of the proteins. The greatest difference in method efficiency arises within basic fractions (17 to 24). Using Agilent's protocol, fractions 17 to 19 resolve only few protein bands, and fractions 20, 21 and 24 contain no visible proteins; most of the basic proteins are resolved within fraction 22 and, to a lesser extent, fraction 23 (Figure 2A). Our in-house method proves superior in this alkaline range, with all the fractions displaying many protein bands, except fractions 19 and 24 which contain no visible proteins (Figure 2B).

Protein depletion within fractions of extreme pH was also observed along a 3-10 pH gradient [20]. The most abundant proteins, such as RuBisCO (large subunit indicated by an arrow in Figure 2), are resolved across several OGE compartments. This was also reported for highly prominent animal proteins such as myosin and actin [19, 21]. The limit of resolving power of OGE when high protein loads are used has been evidenced [32]. Based on the literature, we used settings recommended by the manufacturer to perform the OGE and did not attempt to optimise them. Perhaps plant samples necessitate longer focusing periods and/or higher voltages. Therefore OGE in itself is not resolving enough; however, combined to other analytical techniques such as 2-DE, it greatly improves protein resolution as illustrated below.

The high resolution offered by 2-DE was our third approach to assess which of the two methods employed during OGE was the most efficient in fractionating proteins from wheat leaf samples. In our study, 2-DE was performed using IPG-IEF in the first dimension and SDS-PAGE in the second dimension. For the first dimension, four pH gradients were employed, 3-6, 4-7, 5-8, and 7-10, along 24 cm IPG strips. Following prefractionation and pH measurement, fractions were pooled according to the pH ranges covered by the IPG strips (Table 3). Regardless of the pH range, both Agilent (Figure 3A-E left panel) and our in-house (Figure 3F-J,

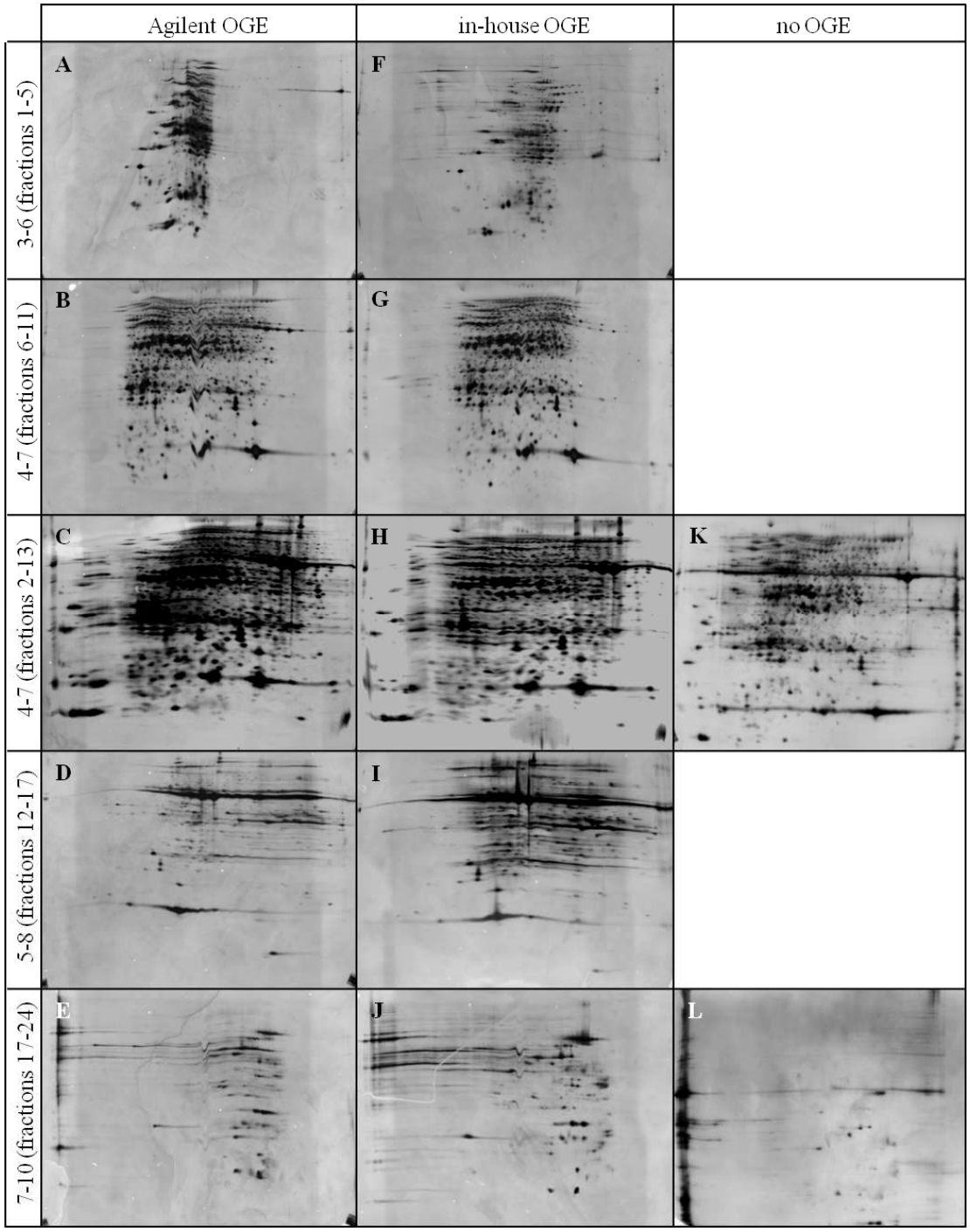

Figure 3. Two-dimensional patterns from leaf samples following either Agilent (A-E) or our in-house (F-J) method along 3-6 (A,F), 4-7 (B-C,G-H,K), 5-8 (D,I) and 7-10 (E,J,L) IPG strips with (A-J) or without (K-L) OGE prefractionation.

middle panel) methods produced excellent two-dimensional patterns bearing many well-resolved spots and a low background noise. A prefractionation using the Agilent method followed by IPG-IEF along a 3-6 pH gradient generated two-dimensional gels displaying incomplete protein focusing as attested by horizontal streaks within medium to high MW range (Figure 3A). All the spots resulting from the in-house method were well-resolved along this acidic gradient (Figure 3F). When fractions 6 to 11 corresponding to 4-7 pH range were pooled and further resolved by 2-DE, the edges of the two-dimensional patterns became spot-depleted (Figure 3B,G), possibly due to inaccurate pH readings. By pooling more fractions (2 to 13), we were able to fully exploit the whole gel area, even if the background noise (dark areas around the spots) increased (Figure 3C,H). Patterns along 5-8 pH range (Figure 3D,I) present horizontal streaks possibly because it corresponds to the focusing range of the most abundant protein of our sample, RuBisCO. Consistent with

1-DE observations, the in-house method seems to improve protein focusing within neutral to basic ranges as can be seen along pH gradients 5 to 8 (Figure 3I) and 7 to 10 (Figure 3J) relative to the patterns produced using the Agilent method (Figures 3E and 3J, respectively).

The primary aim of this paper was to provide an alternative to the Agilent kit. To this end, we did not attempt to optimise OGE solutions or program settings. However, we can anticipate improved protein separation through the optimisation of both the composition of the OGE solution and the OGE run. This warrants further investigation. The results presented here are significant as we estimate that our in-house protocol is ten-fold cheaper than Agilent proprietary method. Being perfectly adjusted to the OFFGEL fractionator, light plasticware equipment (electrodes, tray, well frames, and lids) must be purchased from Agilent Technologies. Although disposable, plasticware can be re-used several times provided pieces are thoroughly cleaned (isopropanol for the electrode, 10% SDS for the rest). Acquiring plasticware consumables through the manufacturer and re-using them will satisfy most laboratory's needs. Focusing solutions are very quick, easy and cheap to prepare; a lab familiar with proteomic procedures will already possess all the necessary chemicals, which must be of electrophoresis grade. Mineral oil or alternatively paraffin oil can be obtained through several suppliers. Although we have used electrode pads supplied by Bio-Rad (too wide we had to re-cut them), pads can be self-made using filter paper. We have not tested IPG strips supplied by manufacturers other than GE Healthcare; however because IPG strip format is standard, we anticipate that any manufacturer will provide compatible material. Thus far, the Agilent kit is amenable to OGE fractionation along 4 to 7 and 3 to 10 linear pH ranges only. For the purpose of this study, we have only tested OGE prefractionation along a linear 3-10 pH range which suited our subsequent 2-DE steps. Whether home-made or manufactured, linear or not, several pH ranges are available. They can be used during OGE to improve the focusing of proteins with extreme or overlapping pI. Only through the use of an in-house protocol can the end-user benefit from such an array of pH ranges.

2. *An OGE prefractionation steps improves 2-DE focusing and sensitivity and allows recovery of proteins of low abundance in dilute samples*

Initial unfractionated leaf samples were also subject to 2-DE along 4-7 and 7-10 pH ranges and used as a reference (Figure 3K-L). Compared to two-dimensional gels obtained from prefractionated samples using OGE, the enrichment in spots and gain in resolution are considerable, particularly within basic ranges (Figure 3). OGE technology relies on soluble- or liquid-phase IEF. To the best of our knowledge, OGE was performed prior to 2-DE only once on human plasma [12]. Introducing a prefractionation step through a liquid-phase IEF device prior to 2-DE has proven a very successful strategy to increase protein coverage and sensitivity in few biological systems such as rodent livers [33, 34], human parasite [35], human cancer [34, 36-38], none dealing with plants. Two-dimensional electrophoresis coupled to mass spectrometry (MS) still constitutes the almost unique platform utilized in plant proteome analysis [39], in particular abiotic stress response [40] which concerns most of plant research. With the gain in MS sensitivity, there is an urgent need in maximising spot resolution on two-dimensional gels in order to achieve the ideal focusing level of one protein *per* spot, as well as to augment the proportion of proteins of low abundance or highly hydrophobic. To date electrophoretic prefractionation appears to be one of the best strategies but has yet to be embraced by the plant proteomic community.

Because OGE proved successful on leaf samples, we applied the technology to apoplast fluid recovered from wheat leaves. Apoplast fluid has a low protein content (ten times less than leaf samples), and, being a subcellular fraction of leaves, are less complex than whole leaf samples. It is also rich in non protein components, such as sugars and organic acids [41]. Apoplastic fluid warrants further studies as it is a dynamic compartment involved in key processes such as defense, signalling, and solute reallocation [42]. Taking into account dilution issues, we have opted for a minimal manipulation of apoplastic fluid through a simple lyophilisation in order to minimise protein loss. Lyophilised apoplast samples were then resuspended in the focusing solution. Although unfractionated apoplast samples produce clean complex one-dimensional profile (lane UA in Figure 4A), the fractions obtained following OGE separation are smeared especially when rich in proteins (fractions 4 to 22). We hypothesise that lyophilised apoplastic fluids comprise too many interfering compounds, and might need further cleaning steps, such as dialysis. Nonetheless, a prefractionation step greatly helps enriching in proteins of low MW and abundance. This enrichment phenomenon was also reported on dilute fungal secretome samples [43]. Two-dimensional gels from prefractionated apoplast samples were produced by pooling more fractions than merely dictated by measured pH values along pH ranges 3-6, 4-7, 5-8, and 7-10 (Table 3 and Figure 4B-E). When compared to unfractionated samples (Figure 4F-G), the enrichment in proteins is obvious despite the high background noise, especially within neutral to basic ranges. Protein content is low in apoplastic fluids, and direct 2-DE without OGE prefractionation is only able to resolve the most prominent apoplastic proteins, which are mainly acidic (Figure 4F) since only few spots are visible along basic gradient (Figure 4G). Patterns spanning acidic to slightly basic gradients displayed many proteins (Figure 4B-D); however, very basic apoplastic proteins remained unfocused (Figure 4E). Because protein enrichment driven by OGE is performed indiscriminately, regardless of abundance, prominent proteins reach amounts that fall outside the detection range of silver nitrate staining and become saturated (very dark areas on Figure 4C in particular). This issue could be resolved by depleting the samples of the most abundant

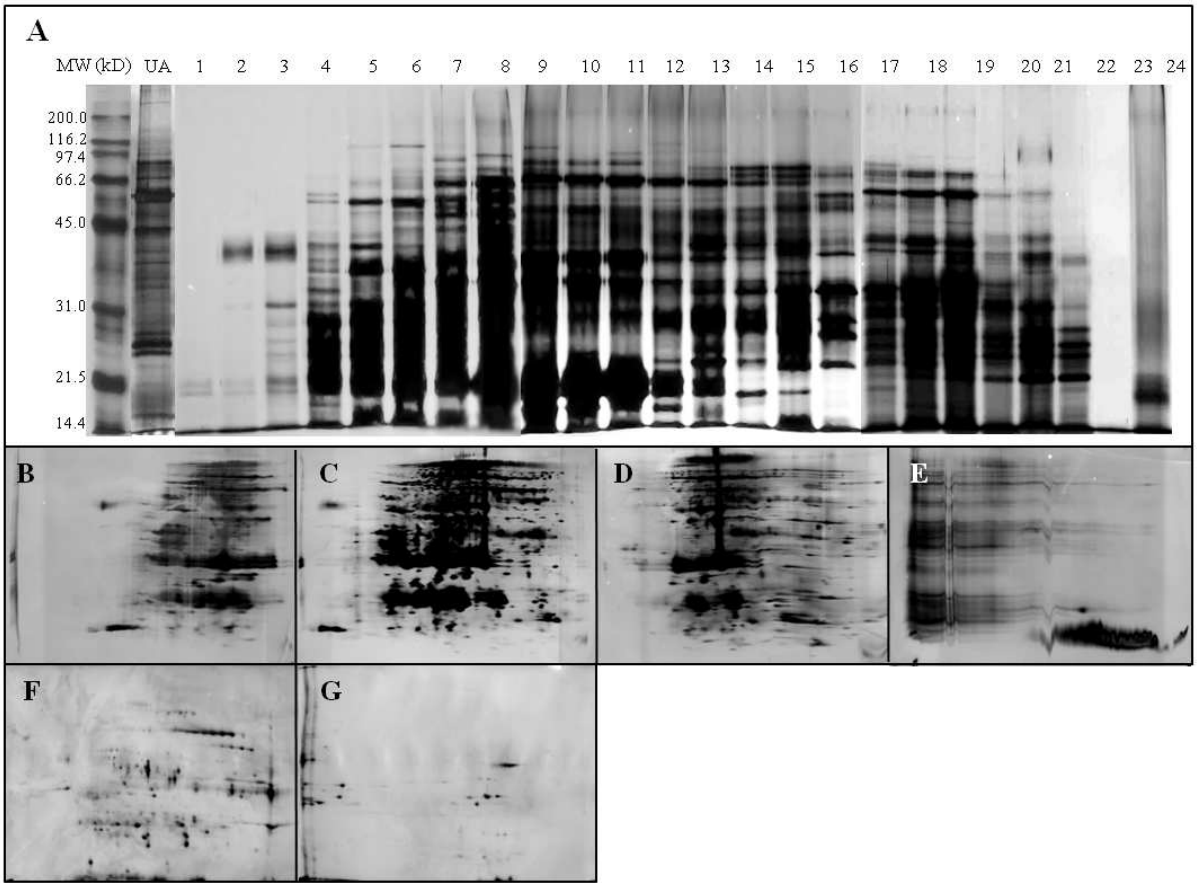

Figure 3. Two-dimensional patterns from leaf samples following either Agilent (A-E) or our in-house (F-J) method along 3-6 (A,F), 4-7 (B-C,G-H,K), 5-8 (D,I) and 7-10 (E,J,L) IPG strips with (A-J) or without (K-L) OGE prefractionation.

proteins. Depletion steps combined to OGE were successfully applied to samples characterised by an enormous concentration range [13, 22, 44].

4. Conclusion

For the first time, proteins from plant tissues were prefractionated using the 3100 OFFGEL fractionator. Two methods were compared by prefractionating proteins from wheat leaves and apoplastic fluids along a 3-10 pH range into 24 fractions. The Agilent method relied on the complete use of the proprietary starter kit which comprised all necessary chemicals and consumables. Our in-house method only differed from the manufacturer's method by the preparation of the focusing solution using our laboratory electrophoresis grade chemicals and the acquisition of IPG strips and electrode pads from other suppliers. The methods were compared and assessed using pH distribution, one- and two-dimensional patterns. Although OGE fractions obtained with the Agilent method better fitted the expected pH distribution than fractions produced using our in-house protocol, it did not affect protein focusing. Indeed both methods yielded excellent electrophoretic profiles of similar quality; spot resolution and number visibly slightly increased with our in-house method. When applied to very dilute samples such as apoplastic fluids, OGE allowed to recover proteins of low abundance. OGE prefractionation should help us discovering novel proteins involved in wheat defense response. We are currently investigating peptide prefractionation on those tissues. Such strategy can be indiscriminately applied to any proteomic project, plant scientists should definitely consider it.

Acknowledgements

This research was supported by the Australian Research Council (DP0986139).

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