

TUTORIAL

Protein extraction from plant tissues for 2DE and its application in proteomic analysis

Xiaolin Wu, Fangping Gong and Wei Wang

State Key Laboratory of Wheat & Maize Crop Science in Henan Province, Synergetic Innovation Center of Henan Grain Crops, College of Life Science, Henan Agricultural University, Zhengzhou, China

Plant tissues contain large amounts of secondary compounds that significantly interfere with protein extraction and 2DE analysis. Thus, sample preparation is a crucial step prior to 2DE in plant proteomics. This tutorial highlights the guidelines that need to be followed to perform an adequate total protein extraction before 2DE in plant proteomics. We briefly describe the history, development, and feature of major sample preparation methods for the 2DE analysis of plant tissues, that is, trichloroacetic acid/acetone precipitation and phenol extraction. We introduce the interfering compounds in plant tissues and the general guidelines for tissue disruption, protein precipitation and resolubilization. We describe in details the advantages, limitations, and application of the trichloroacetic acid/acetone precipitation and phenol extraction methods to enable the readers to select the appropriate method for a specific species, tissue, or cell type. The current applications of the sample preparation methods in plant proteomics in the literature are analyzed. A comparative proteomic analysis between male and female plants of *Pistacia chinensis* is used as an example to represent the sample preparation methodology in 2DE-based proteomics. Finally, the current limitations and future development of these sample preparation methods are discussed. This Tutorial is part of the International Proteomics Tutorial Programme (IPTP17).

Received: June 17, 2013
Revised: December 3, 2013
Accepted: December 10, 2013

Keywords:

2DE / Plant proteomics / Sample preparation / Tutorial



Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Historical background

2DE with IPGs is one of the most powerful tools for large-scale protein separation and quantification [1]. In 2DE, proteins are separated according to pI in the first-dimensional IEF and are then separated according to molecular weight (M_r) in the second-dimensional SDS-PAGE [2]. Currently, 2DE combined with MS is the major platform for proteomics [3]. The aim of most 2DE analyses is to maximize the number

of polypeptides that can be resolved, especially for comparative proteomics, which generally involves looking for minor differences between experimental and control samples [4].

In practice, however, the result of 2DE often suffers problems in the coextraction of nonprotein cellular components that adversely affect protein migrations [5], thus resulting in streaking and smearing and in a reduction in the number of distinctly resolved protein spots. This problem is more prominent in plant tissues that have low cellular protein contents but high amounts of proteases and interfering compounds [6]. Therefore, protein extraction before 2DE is a very crucial step in the methodology of plant proteomics.

The ideal extraction method should reproducibly capture the most comprehensive repertoire of proteins possible while minimizing protein degradation and nonprotein contamination [7]. Over the past 30 years, with the constant improvement of 2DE technology, many sample preparation methods for plant tissues have been developed for enhanced 2DE and

Correspondence: Dr. Wei Wang, State Key Laboratory of Wheat & Maize Crop Science in Henan Province, Synergetic Innovation Center of Henan Grain Crops, College of Life Science, Henan Agricultural University, Zhengzhou 450002, China
E-mail: wangwei@henau.edu.cn
Fax: +86 371 63579690

Abbreviations: 2-ME, 2-mercaptoethanol; PVPP, polyvinylpolypyrrolidone; TCA, trichloroacetic acid

proteomic analysis [e.g., 1, 8–17]. These methods are typically based on trichloroacetic acid (TCA)/acetone precipitation and phenol extraction.

A one-step protocol, that is, denaturing extraction in lysis buffer [18], was recognized as unsuitable for plant materials. In the 1980s, a precipitation step was introduced into most plant methods to concentrate the proteins and to separate them from interfering compounds [8–10, 19–24]. In particular, the TCA/acetone precipitation developed by Damerl et al. [8] is very popular and performs efficiently with a large variety of plant tissues. It directly precipitates total proteins from homogenized tissue or cells with TCA and 2-mercaptoethanol (2-ME) in cold acetone [8]. Méchin et al. [4] described in detail the methodology of TCA/acetone precipitation for plant proteomics.

Phenol extraction was also developed in the 1980s [10, 20, 24]. It can be traced back to the 1950s, when phenol was found to be effective for extracting proteins from aqueous solutions [25]. In plants, Schuster and Davies [20] first used phenol extraction in pea epicotyls. Hurkman and Tanaka [24] later used Schuster and Davies's methodology [20] to solubilize the membrane proteins of barley roots for 2DE. In this method, proteins are solubilized in phenol, with or without SDS, and are subsequently precipitated with methanol and ammonium acetate, followed by resolubilization in the IEF buffer. However, before 2000, this method was not as widely used as TCA/acetone precipitation. In 2003, Wang et al. [12] developed a TCA/acetone/phenol extraction method, which integrates TCA/acetone precipitation with phenol extraction, to effectively extract proteins from evergreen olive leaves for 2DE analysis. Since then, significant attention has been paid to the powerful effect of phenol extraction in recalcitrant plant tissues (e.g., fiber-rich tissues and fruits). At present, phenol-based methods are also commonly used in plant proteomics, and in many cases, these methods are more powerful than TCA/acetone precipitation. The methodology of phenol extraction has been described in detail in [15, 17].

This tutorial highlights the guidelines that need to be followed to perform an adequate protein extraction prior to 2DE, focusing on the applications of the TCA/acetone precipitation and phenol extraction of total proteins from plant tissues. We have attempted to minimize the overlap with published excellent reviews and tutorials [e.g., 2, 7, 26–29].

2 Basic concepts

2.1 Interfering compounds

Plant tissues contain large amounts of compounds that severely affect the performance of protein extraction and separation in 2DE gels. The common interfering compounds include phenolics, terpenes, pigments, organic acids, lipids, proteolytic and oxidative enzymes, ions, nucleic acids, and polysaccharides. In particular, plant phenolics include approximately 8000 types of naturally occurring compounds,

such as phenols, flavonoids, stilbenes, tannins, and lignins [30], all of which share a common aromatic ring bearing at least one hydroxyl substituent [31]. These compounds mainly accumulate in soluble forms in vacuoles that occupy a large volume of the cell. The existence of secondary metabolites displays species/tissue specificity and varies with age or developmental stage. Secondary metabolites are more abundant in adult, green tissues than in young, etiolated tissues [9]. However, nucleic acids are rich in rapidly dividing cells and young tissues (e.g., root tips).

Phenolics can build irreversible complexes with proteins, forming very strong hydrogen bonds with the oxygen atoms of peptide bonds or condensing with -SH and -NH₂ groups. The oxidation of phenolics by phenoloxidases and peroxidases results in streaking and generates artifactual spots on 2DE gels [32]. Lipids bind proteins via hydrophobic interactions, affecting their charge and relative *M_r*. In many cases the lipid–protein complex is insoluble in aqueous solution, resulting in a failure to enter the first-dimension gel [33]. Polysaccharides can interfere with IEF by obstructing gel pores [7]. Nucleic acids can bind proteins through electrostatic interactions, preventing IEF. High *M_r* nucleic acids can additionally clog the pores of the acrylamide matrix [11]. Other substances, such as endogenous ions, nucleotide metabolites, and phospholipids, which are present in the cell lysates, are often negatively charged, resulting in poor focusing toward the anode [5].

The elimination of phenolics and other interfering compounds is a prerequisite for satisfactory protein extraction from plant tissues. Two strategies are generally used to remove the interfering compounds before 2DE: removal before protein extraction and removal during and after protein extraction. In both strategies, the oxidation of phenolic compounds may be prevented with the use of water-soluble PVP or water-insoluble polyvinylpyrrolidone (PVPP) [15, 34], DTT or 2-ME, sodium ascorbate [22], and thiourea [23] while performing the tissue extraction.

In the first strategy, finely ground tissue powders are subjected to 10% (w/v) TCA/acetone (plus DTT or 2-ME) precipitation. After extensive organic solvent cleanups, the tissue pellet should ideally be white or very light colored, indicating the removal of the majority of secondary metabolites (e.g., phenolics and pigments). Afterwards, the air-dried acetone powder is extracted in aqueous buffers. This strategy can also greatly reduce the volume of the starting materials, facilitating downstream manipulations. The pretreatment of leaf tissue powder with acetone removes not only pigments, such as chlorophylls, but also large amounts of various phenolics that can interact with proteins and create artifacts, particularly in the presence of urea [19]. In the case of lipid-rich tissues, such as seed tissue, excess lipids can be removed with acetone and chloroform [33].

In the second strategy, the proteins are directly extracted from the plant tissue in an aqueous buffer, and the resultant extract is subjected to TCA/acetone precipitation or phenol extraction. The extraction buffer usually contains EDTA, DTT or

2-ME, a protease inhibitor cocktail, and PVPP. In particular, nucleic acids, ions, and polysaccharides can be effectively removed via phenol extraction [29]. However, the contaminants coprecipitated with proteins are often difficult to remove in subsequent organic solvent cleanups; therefore, this strategy is usually applied in relatively easy tissues, such as etiolated shoots and young tissues (e.g., root tips).

2.2 Tissue disruption

Plant cells consist of the outermost walls that are mostly composed of cellulose and its derivatives (including large anionic pectins) [35]. Young plant cells are surrounded with a primary cell wall; in some plant cell types, a rigid secondary cell wall is present between the plant cell and the primary wall after the cell development. In woody plants, the content of lignin and related compounds (especially in roots and stems) is even higher than in other plant species making the disruption of the cell wall problematic [36]. The disruption of the cell wall and protein release is crucial for the following extraction steps. Various chemical and physical techniques are used to destroy the cell wall, such as lysis buffer, sonication, freeze-thawing, and high-speed blending [28].

A common plant tissue disruption step is pulverizing materials in liquid N₂ with a mortar and pestle (or with the help of quartz sands), particularly for fiber-rich crop tissues [17, 29]. This practice can also minimize proteolysis and protein modifications occurring during tissue disruption. To ensure complete tissue disruption, use a small amount of starting material (e.g., 0.2–0.5 g of fresh weight). The yield of the total proteins extracted from a plant tissue depends greatly on the fineness of the tissue powder.

Alternatively, homogenizing the plant material in TCA/acetone in a device that generates strong shearing forces (e.g., a polytron or glass homogenizer) [37] also produces a fine powder. Young tissues (e.g., root tips, watery fruit tissues, and developing seeds) can be directly homogenized in cold TCA/acetone.

2.3 Protein precipitation

Cell or tissue extracts of proteins often contain interfering substances that must be removed. A number of protein precipitation methods [38] are available for this purpose. The precipitation step is regarded as a necessity when handling recalcitrant plant tissues to obtain high-quality 2DE profiling [13]. TCA/acetone precipitation and phenol extraction followed by methanol precipitation are the most frequently used techniques for total protein extraction in plant proteomics. Both methods can concentrate the sample and simultaneously remove small interfering compounds before 2DE.

2.3.1 TCA/acetone precipitation

TCA/acetone precipitation, originally developed by Damerval et al. [8], is based on protein denaturation under acidic and/or

hydrophobic conditions that help concentrate proteins and remove contaminants. Méchin et al. [4] recently described a protocol in detail, from protein precipitation and denaturation to resolubilization in a solution for subsequent IEF. The steps of the TCA/acetone precipitation have many modifications (Table 1). For example, in its original version [8], tissue powder and pellet were kept each time in 10% TCA/acetone (plus 0.07% 2-ME) for 45 min at –18°C; on a small scale, the incubation time can be shortened to 5–10 min, or the sample can be directly centrifuged [43].

The main steps of TCA/acetone precipitation involve the following (Fig. 1): (i) prepare a fine tissue powder; (ii) homogenize the tissue powder in TCA/acetone (optional); (iii) wash the powdered tissue in TCA/acetone and then acetone (typically, after two washes each with TCA/acetone and acetone, the powdered tissue should ideally be white or light-colored); and (iv) extract the powdered, air-dried tissue in the buffer of choice (e.g., 2DE lysis buffer). The major caveat is keeping the samples at a low temperature during extraction, including a reducing agent (e.g., DTT or 2-ME) in cold organic solvents, and not overdrying the pellet before resolubilization. To troubleshoot TCA/acetone precipitation, see Isaacson et al. [15].

Alternatively, proteins can be first extracted from plant tissue with various extraction media, followed by precipitation of the protein extract with acetone [44], aqueous TCA [45], or TCA/acetone [46, 47].

The TCA/acetone precipitation method allows the efficient extraction of total proteins for a large variety of plant tissues, especially young tissues. However, it was found not to be the best choice for more complex plant tissues [12, 13, 48]. For example, in grape berry, Vincent et al. [49] found that TCA/acetone precipitation, with the lowest protein content, gave the poorest results with few ill-resolved spots, compared to phenol-based protocols. Because it is less time consuming and easier to perform than the phenol-based protocols, TCA/acetone precipitation is recommended as a starting protocol for plant proteomic analyses.

TCA/acetone precipitation has the following advantages: (i) it is easy to perform, even for a very large volume of plant samples; (ii) it removes many compounds (particularly ions, lipids, pigments, phenolics, and terpenoids) that interfere with IEF from the samples more effectively than either TCA or acetone alone; (iii) it precipitates proteins and simultaneously inactivates components involved in protein degradation and modification, such as proteases, phenoloxidases, and peroxidases [8, 9, 48]; and (iv) it is also valuable for the enrichment of very alkaline proteins (e.g., ribosomal proteins) from total cell lysates [50].

Nonetheless, the limitations of TCA/acetone precipitation include the following: (i) the precipitated proteins are often difficult to dissolve [13, 51], therefore, a strong resolubilization buffer must be used with this method; and (ii) some polymeric contaminants are often coextracted, which is a particular problem with tissues rich in soluble polysaccharides and polyphenols from cell walls. Compared to acetone precipitation, several spots in 2DE were lost after TCA/acetone

Table 1. The modifications of TCA/acetone precipitation

Plant material	First precipitation	Incubation	Centrifugation	Wash	Ref.
Wheat seedling	10% TCA, 0.07% 2-ME in acetone	45 min at -18°C	$35\,000 \times g$ for 15 min	Once, acetone for 1 h at -18°C	[8]
Maize leaves	10% TCA, 0.07% 2-ME in acetone	1 h at -18°C	$35\,300 \times g$ for 15 min	Once, acetone for 1 h at -18°C	[9]
<i>Arabidopsis thaliana</i> leaves and stems	10% TCA, 0.07% DTT in acetone	45 min at -20°C	$35\,000 \times g$ for 15 min	Once, acetone for 1 h	[39]
Banana leaves	20% TCA, 0.2% DTT in acetone	Overnight at -20°C	$16\,000 \times g$ for 30 min	Twice, acetone incubation for 1 h at -20°C	[13]
Beech leaves and roots, spruce needles	10% TCA, 20 mM DTT in acetone	Overnight at -20°C	$26\,000 \times g$ for 30 min at 4°C	Twice, acetone incubation for 1 h at -20°C each	[36]
Papaya leaves	10% TCA, 0.07% 2-ME in acetone	Overnight at -20°C	$16\,000 \times g$ for 30 min at 4°C	Twice, acetone for 1 h at -20°C each	[40]
Cabbage leaves and carrot roots	10% TCA, 0.07% 2-ME in acetone	1 h at -20°C	$20\,000 \times g$ for 15 min at 4°C	Thrice, acetone for 1 h at -20°C	[41]
Common bean leaves	10% TCA, 1% DTT in acetone	At least 2 h at -20°C	$13\,000 \times g$ for 15 min at 4°C	Thrice, acetone	[42]

In wash step, acetone contains the same reducing reagent (same concentration) as in the first precipitation.

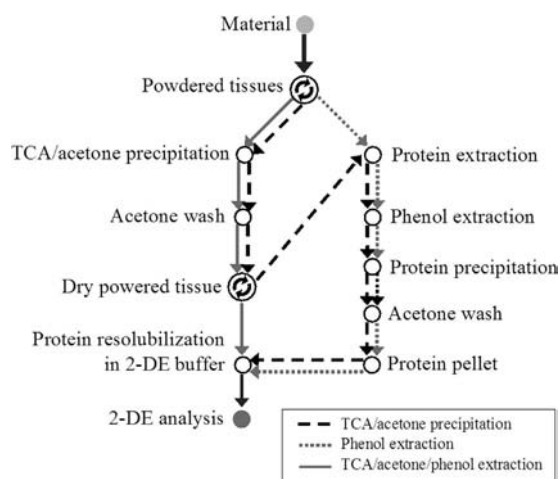


Figure 1. Three protocols for sample preparation of plant tissues in proteomic analysis. It should be noted that these protocols may be modified to achieve optimal results for different plant tissues and they are often complementary.

precipitation, particularly large proteins, but some spots were enriched (Fig. 2).

2.3.2 Phenol extraction and methanol precipitation

Phenol extraction is an alternative method to classical TCA/acetone precipitation. It is often performed according to the methodology of Hurkman and Tanaka [24]. The main steps of phenol extraction are shown in Fig. 1. Briefly, proteins are first extracted with an aqueous buffer (with or without SDS) and then mixed with the buffered phenol (pH 7.8–8.8,

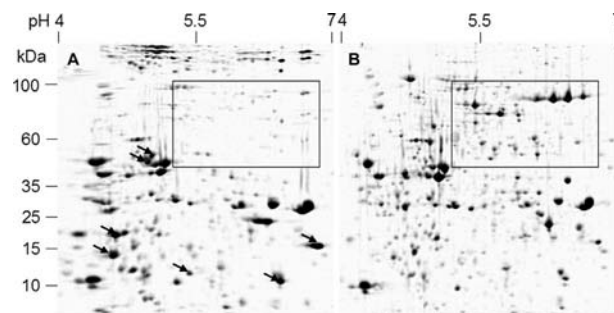


Figure 2. Comparison of 2DE maps produced using TCA/acetone precipitation and acetone precipitation. The fifth leaves of maize seedlings were ground to a fine power in liquid nitrogen and then rinsed with (A) TCA/acetone and (B) acetone. The final proteins were dissolved in the same 2DE rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, and 2% IPG buffer). Equal protein loading (approximately 500 μg) was resolved by IEF using 11 cm linear IPG strips (pH 4–7) and then separated using 12.5% SDS/acrylamide gels. Gels were stained with CBB R350. Many proteins (particularly large proteins, rectangle) were lost after TCA/acetone precipitation. Arrows indicate protein spots which were enriched by TCA/acetone precipitation.

available commercially), causing proteins to denature and dissolve in the phenol phase, whereas other water-soluble substances (e.g., salts, nucleic acids, and carbohydrates) remain in the aqueous phase. Proteins in the phenol phase are thus purified and concentrated simultaneously by subsequent methanol precipitation [12,15]. Proteins in the phenolic phase are precipitated with ammonium acetate in methanol. The precipitated proteins are then resuspended in 2DE lysis or rehydration buffer. In particular, the coextracted phenolic substances are separated from the proteins after methanol

precipitation. Faurobert et al. [52] described a tried and tested phenol extraction protocol adapted for 2DE and further proteomic studies. Carpentier et al. [13] optimized the phenol extraction protocol for small amounts of tissue, which is essential when the study material is limited.

Many modified phenol extraction methods exist (Table 2). For example, Hurkman and Tanaka [24] used a detergent-free buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM DTT, 0.1 M KCl, 2% 2-ME, and 2 mM PMSF) to solubilize plant proteins, whereas Wang et al. [12, 43] later used a SDS-containing buffer to increase protein extraction. Some researchers [12, 13, 15, 43] include sucrose (30% or 0.7 M) in the aqueous extraction buffer to ensure that the aqueous phase is heavier than buffered phenol. After phase separation by centrifugation, the phenol phase is “pushed” on top, facilitating the extraction of the phenol phase. The protein pellet from phenol extraction should look white and a yellowish pellet indicates coprecipitation of phenolics. Pulverizing plant tissues with PVPP (0.05 g/g tissue) or plus 1% w/v PVPP in the lysis buffer prior to phenol extraction improved protein extraction (see review, [29]).

The important points for the successful use of phenol extraction include the following: (i) keep samples at a low temperature during the first extraction step; (ii) pipet out the correct phenolic phase after phase separation; and (iii) adjust the pH of the phenol solution and protein extract if appropriate because only after satisfying the basic conditions can the proteins be partitioned into the phenol phase. Bromophenol blue is compatible with phenol and is a good pH indicator (blue at $\text{pH} \geq 7$). For typical steps and troubleshooting of phenol extraction, the readers may refer to [12, 13, 43, 48], in particular [15, 17, 32, 52].

The advantages of phenol extraction include the following: (i) it is more effective for producing high-quality protein samples than TCA/acetone precipitation; (ii) it also inactivates enzymes without heating, thereby minimizing protein degradation resulting from endogenous proteolytic activity proteolysis during extraction [20]; and (iii) it allows the removal of polysaccharides, ions, and nucleic acids that are partitioned into the aqueous phase.

Nonetheless, phenol extraction has some limitations: (i) some specific proteins may be lost after phenol extraction [13], especially large glycoproteins [56], although a different report showed that the enhanced extraction of glycoproteins in three types of fruits (i.e., banana, avocado, and orange) using phenol extraction [48]; (ii) its toxicity and time-consuming nature should be considered when a sample preparation procedure is being designed; and (iii) it is often not able to produce high-quality 2DE profiles in recalcitrant tissues containing large amounts of phenolic compounds (e.g., olive leaves and fruits) [12].

Overall, the phenol-based protocols are superior to the TCA/acetone methods, showing larger protein yields and greater spot resolution on 2D gels, but they are also complementary to TCA/acetone precipitation [13, 48]. The phenol-based protocols more likely provide satisfactory results in

plant tissues rich in interfering compounds, such as leaves [13], fruits [13, 48, 49, 53, 57–59], and wood [60] as well as in cell-wall fractions [61]. There is a small but significant difference between the protein pattern of the TCA/acetone precipitation and phenol extraction [13, 48, 62]. These differences may result from the extra phenol extraction step (associated with phase partitioning) and the precipitation method.

2.3.3 TCA/acetone/phenol extraction

Wang et al. [12, 43] combined phenol extraction with TCA/acetone precipitation before 2DE for the protein extraction of recalcitrant plant tissues. This protocol was recently described in detail [17]. It holds the merits of both TCA/acetone precipitation, which effectively removes non-protein compounds, and phenol extraction, which selectively dissolves proteins, to facilitate the most effective purification of proteins. The protocol first involves TCA/acetone precipitation and then SDS extraction followed by phenol extraction (Fig. 1). With SDS extraction, proteins precipitated by TCA/acetone can be fully resolubilized and then purified further by phenol extraction. Originally, the powdered tissue sample was resuspended in the mixture (1:1, v/v) of phenol (Tris-buffered, pH 8.0) and dense SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% 2-ME) [12]. Alternatively, proteins can be extracted from the tissue powder using the SDS buffer, and protein extract is then subjected to phenol extraction [63].

The TCA/acetone/phenol extraction protocol was originally designed to extract proteins from adult evergreen olive leaves that are notoriously recalcitrant to common protein extraction methods due to the large amounts of phenolic compounds [64]. For olive leaves, phenol extraction combined with ongoing TCA/acetone precipitation is more efficient than either method alone. This protocol has been successfully used in various tissues from different plant species [e.g., 65–71]. It provides an enhanced 2DE-based proteomic analysis of a range of recalcitrant tissues for proteomic analyses [12, 43, 48].

This protocol is capable of producing more high-quality proteins than either TCA/acetone precipitation or phenol extraction alone. Maldonado et al. [72] evaluated the three above-mentioned protocols (TCA/acetone precipitation, phenol extraction, and TCA/acetone/phenol extraction) for *Arabidopsis thaliana* leaf proteome analysis by 2DE. Their results showed that the TCA/acetone/phenol protocol provided the best results in terms of spot focusing, resolved spots, spot intensity, unique spots detected, and reproducibility, and there were no statistically significant differences in protein yield among the three protocols. Similarly, Vincent et al. [49] and Jorin et al. [73] reported that the TCA/acetone/phenol protocol resulted in improved 2DE gels with various plant tissues in terms of the number of spots resolved. Compared to TCA/acetone precipitation and phenol extraction,

Table 2. The selected applications and modifications of phenol extraction in plant proteomics

Plant material	Extraction buffer	Phenol ^{a)}	Phase incubation and separation	Wash step	Reference
Plasma membrane of barley roots	0.5 M Tris, 30 mM HCl, 0.7 M sucrose, 50 mM EDTA, 0.1 M KCl, 2% 2-ME, 2 mM PMSF	Water saturated phenol	10 min at RT, 6000 × g for 10 min at RT	Once with the extraction buffer	[24]
Olive leaves and fruits	30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% 2-ME	Tris buffered phenol (pH 8.0)	Vortexing 30 s, 10 000 × g for 3–5 min at RT	None	[12, 43]
Tomato leaves, fruits (avocado, banana and orange)	0.5 M Tris-HCl, pH 7.5, 1% PVPP, 0.1 M KCl, 0.7 M sucrose, 0.5 M EDTA, 1 mM PMSF, 2% 2-ME	Tris buffered phenol (pH 7.5)	30 min at 2°C, 10 000 × g for 30 min	Twice or thrice, with the extraction buffer	[48]
Banana leaves and meristems	50 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.1 M KCl, 1% DTT, 30% sucrose	Tris buffered phenol (pH 8.0)	15 min at 4°C, 6000 × g for 3 min at 4°C	Once, using the extraction buffer	[13]
Grape roots and leaves	5% w/w PVPP; 0.5 M Tris-HCl, pH 8, 0.7 M sucrose, 10 mM EDTA, 0.4 % 2-ME, 4 mM ascorbic acid, 0.2 % Triton X-100, 1 mM PMSF, 1 μM Leupeptin, 0.1 mg/mL Pefabloc	Tris buffered phenol (pH 8.0)	2 h at 4 °C, 5000 × g for 20 min at 4°C	None	[53]
Maize root tips	0.5 M Tris-HCl, pH 7.5, 0.7 M sucrose, 0.1 M KCl, 50 mM EDTA, 2% 2-ME, 1 mM PMSF	Tris buffered phenol (pH 7.5)	30 min at 4°C, 5000 × g for 30 min at 4°C	Once, using the extraction buffer	[15]
<i>Pteris vittata</i> roots	0.5 M Tris-HCl, pH 7.5, 0.7 M sucrose, 50 mM EDTA, 0.1 M KCl, 10 mM thiourea, 2% 2-ME, and 1% protease inhibitor	Tris buffered phenol (pH 8.8)	45 min	Once, using the extraction buffer	[54]
Kiwi shoots	0.1 M Tris-HCl, pH 8.0, 0.5 M EDTA, 30% sucrose, 1% PVPP, 0.1 M KCl, 2% SDS, 1 mM phenylmethanesulfonyl fluoride, 5% 2-ME	Ice-cold Tris-HCl buffered phenol (pH 8.0)	30 s, 17 000 × g for 5 min at 4°C	None	[55]

a) Protein extracts are directly, or after clarified with centrifugation, mixed with buffered phenol (1: 1, v/v).
RT: room temperature.

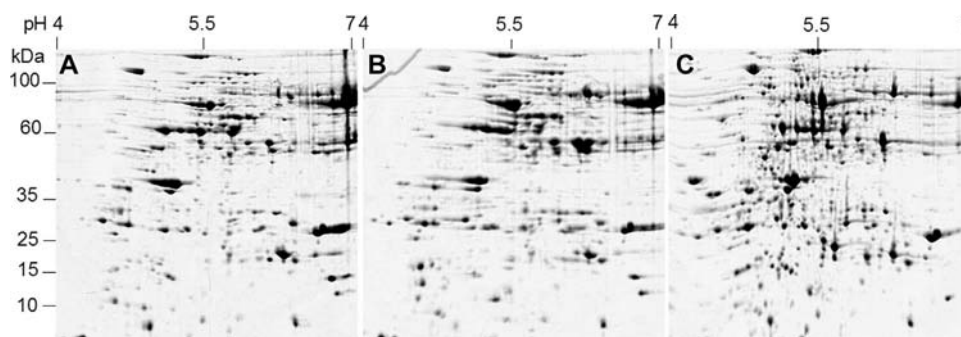


Figure 3. Comparative 2DE analysis of maize leaves proteins using three extraction methods. (A) TCA/acetone/phenol extraction, (B) phenol extraction, (C) TCA/acetone precipitation. TCA/acetone/phenol extraction produced better 2DE maps than phenol extraction and TCA/acetone precipitation. Proteins (approximately 500 μ g) were resolved by 2DE as described in Fig. 2 legends.

TCA/acetone/phenol extraction produced better 2DE results in the case of maize leaves (Fig. 3).

As this protocol combines TCA/acetone precipitation and phenol extraction, it is somewhat time-consuming. Additionally, small amounts of protein may be lost due to the many steps involved, but this issue can be remedied by performing several parallel extractions. In addition, this protocol can combine with sample prefractionation methods and improve the detection of low abundance proteins [74, 75].

2.4 Protein solubilization

Before 2DE, the proteins from the sample must be fully solubilized. An incomplete solubilization will result in protein losses. This step strongly affects the quality of the final results and thus determines the success of the entire experiment.

Because the first step of 2DE is IEF, the proteins must maintain their own charges in the sample buffer. Two excellent reviews [1, 7] describe the general guidelines and common cocktails for protein solubilization. In general, the sample buffer must dissolve as many proteins as possible and split all noncovalently bound protein complexes into subunits [11]. Furthermore, it must prevent aggregation and keep proteins soluble during the 2DE separation. Finally, it should denature all proteases.

Sample solubilization is usually performed in a buffer containing chaotropes (typically a urea/thiourea mixture) [76–78], nonionic and/or zwitterionic detergent (NP-40 or CHAPS) [79], a reducing agent (DTT or 2-ME) [80, 81], carrier ampholytes, and, depending on the type of sample, protease inhibitors [81, 82] (Table 3). A typical lysis buffer for plant protein samples in 2DE analysis consists of 7 M urea, 2 M thiourea, 20 mM DTT, 2–4% CHAPS, and 2% carrier ampholytes of the corresponding pH range [5, 15, 43, 90]. However, there is no definite rule for selecting the appropriate solubilization cocktail for a given sample: lysis buffer selection is mostly an empirical process [36].

In addition, proteins resulting from TCA/acetone precipitation are often difficult to completely resolubilize, thus a prolonged incubation (1 h) of the protein pellets in the lysis

buffer with shaking is recommended. Before 2DE, the protein extract needs to be clarified by centrifugation to remove insoluble materials that may clog the gel pores.

3 Current uses of the sample preparation approaches in plant proteomics

With the availability of the complete genome sequence of many species (e.g., *Arabidopsis*, rice, maize, grapevine), plant proteomics has fully entered the era of functional genomics. As a valuable tool to identify new genes and a tool for physiological and genetic studies in plants [91], tremendous progress in plant proteomics driven by MS techniques has been made in the last decade [92].

We selected the original articles published in the *Journal of Proteomics* as examples to address the current uses of these sample preparation approaches in plant proteomics. From January 2008 to May 2013, a total of 1037 articles were published in the *Journal of Proteomics*, 153 of which are related to plant biology. Furthermore, only 73 articles consider proteomic analyses, involving 45 plant species, such as the model plants *A. thaliana*, rice, and maize, and many nonmodel crops (e.g., soybean, sugar beet, sorghum, and pea), fruit plants (e.g., peach, papaya, strawberry, and orange), and woody plants (e.g., poplar, Holm oak, and spruce). Obviously, the full potential of proteomics is far from being fully exploited in plant biology compared with other organisms, mainly humans and yeast. In addition, in the mentioned 73 articles, 70 are based on 2DE, and only three use gel-free methods. Thus, 2DE remains the major platform of plant proteomics at present.

Most of the 70 articles report the comparative proteomic profiling of specific tissues with the intention of identifying differentially expressed proteins linked to various physiological and stress responses or that might account for the phenotypic differences observed. Total protein is extracted using four methods: direct precipitation of protein extracts with acetone or TCA precipitation (10), TCA/acetone precipitation (31), phenol extraction (21), and TCA/acetone/phenol extraction (8), followed by resolubilization in a 2DE

Table 3. 2DE rehydration buffers used in protein solubilization in proteomic analysis

Plant material	Urea (M)	Thiourea (M)	Reducing agent	Detergent	Ampholyte (pH)	Other	Ref.
Maize endosperm	5	2	20 mM DTT, 5 mM TCEP	2% CHAPS, 2% SB3-10	0.25% (3–10)		[81]
Banana leaves	6	2	0.28% DTT	0.5% CHAPS	0.5% (3–10)	BrB 10% glycerol	[13]
Fruits (avocado, banana, 7 and orange)		2	10 mM DTT	4% CHAPS	1% (4–7)		[48]
<i>Pistacia chinensis</i> leaves 7 and woody stems		2	20 mM DTT	4% CHAPS	2% (4–7)	BrB	[83]
Cotton fiber	7	2	40 mM DTT	4% CHAPS	1% (4–7)		[84]
<i>Pisum sativum</i> leaves 7 and roots		2	75 mM DTT	2% CHAPS, 1% Triton-X 100, 1% SB-12	0.5% (3–10)	BrB 6% glycerol, 5% 2-propanol	[47]
Peach buds	7	2	0.4% DTE	2% CHAPS	0.2% (3.5–10)	Protease inhibitors	[85]
Papaya leaves	8		0.2% DTT	2% CHAPS	0.5% (3–10)	BrB	[40]
Soybean leaves	8	2	2 mM TBP	5% CHAPS	0.4% (3–10)		[86]
Tomato anthers	8		20 mM DTT	2% CHAPS	2% (3–10)	BrB	[87]
<i>Agapanthus praecox</i> flowers	8		15 mM DTT	2% CHAPS	5% (4–7)	BrB	[88]
<i>Arabidopsis thaliana</i> leaves	9		100 mM DTT	4% CHAPS, 0.5% Triton × 100	2% (3–10)	BrB	[72]
Apricot fruit	9		20 mM DTT	4% CHAPS, 0.5% Triton × 100	1% (3–10)		[89]

BrB: bromophenol blue, with a concentration of 0.001–0.01%; TBP: tributylphosphine.

rehydration buffer (Table 4). Obviously, TCA/acetone precipitation and phenol extraction are widely used in 2DE for plant proteomics. In some cases of plant species not studied before, these methods have to be adapted and further optimized for the proteomics analysis of a specific tissue.

4 Working example

The working example is taken from a recent publication on the proteomic identification of differentially expressed proteins between male and female plants in Chinese pistache (*Pistacia chinensis* Bunge) [83]. This plant is a deciduous, wind-pollinated, large shrub or small tree in the cashew family. It is widely planted in China for biodiesel oil due to the high seed oil content (35–50%, [115]). Thus, female plants of *P. chinensis* have higher economic values than male plants. At present there are no reliable methods for sex determination in the juvenile stage of this species. Therefore, a comparative proteomic approach was used to identify differentially expressed proteins between both sexes to develop sex-linked molecular markers in *P. chinensis*.

In this example, the following items are considered regarding sample preparation:

- (1) Sample used for comparative analysis. Leaf and stem, rather than reproductive organs/tissues, were used and sampled from known-sex adult plants (10- or 40-year-old),

because differentially expressed proteins in reproductive organs/tissues may not be suitable for the sex determination of siblings.

- (2) Tissue disruption. The leaves and stems of *P. chinensis* are rich in fiber and difficult to disrupt, especially the stem. Before disruption, the stem was manually separated into the xylem and phloem, which were then cut into pieces. All samples were pulverized into a fine powder in liquid N₂ with a mortar and pestle.
- (3) Sample preparation method. In the preliminary experiments, acetone precipitation, TCA/acetone precipitation, and phenol extraction did not produce good results for the 2DE analysis. The TCA/acetone/phenol extraction protocol was found to work well in this case. As described in Fig. 1, the dry tissue powder is used for protein extraction. In our hands, an equal amount of dry tissue powder always produces almost the same protein yields when performed in parallel, which facilitates paired comparative 2DE analysis.
- (4) Phenol extraction and methanol precipitation. Briefly, the tissue powder was homogenized in the extraction buffer (0.1 M Tris-HCl, pH 8.8, 2% SDS, and 0.1 M DTT, 1:10 g/mL) in a mortar. The clarified supernatant was extracted with an equal volume of buffered phenol (pH 8.0). After phase separation, the phenolic phase was precipitated with five volumes of cold methanol containing 0.1 M ammonium acetate overnight (–20°C). The protein was recovered by centrifugation and washed twice with

Table 4. The applications of protein precipitation methods in 2-DE based plant proteomics in the selected literature

Protocol	Material	Species	Selected reference	Note
Acetone or TCA precipitation of protein extracts (10)	Seeds	Wheat (<i>Triticum aestivum</i>)	[93]	Used in simple tissues and often resulting in poor 2-DE profiling, except for a few examples, see [93]
	Roots	Douglas fir (<i>Pseudotsuga menziesii</i>)	[94]	
	Leaves	<i>Arabidopsis thaliana</i>	[95]	
	Algal cells	Synechocystis	[96]	
TCA/acetone precipitation (31)	Roots	Rice (<i>Oryza sativa</i>)	[97]	Used in a large variety of tissues from various species (e.g., crops, vegetables, and trees), but often resulting in poor 2-DE profiling with few spots, probably due to incomplete resolubilization.
		Soybean (<i>Glycine max</i>)	[98]	
		Poplar (<i>Populus tremula</i>)	[99]	
	Leaves	Cabbage (<i>Brassica oleracea</i>)	[41]	
		Rice (<i>O. sativa</i>)	[100]	
		<i>Solidago canadensis</i>	[101]	Not the best choice for more complex plant tissues
	Seeds	Soybean (<i>G. max</i>)	[102]	
	Flowers	<i>Agapanthus praecox</i>	[88]	
		Sugar beet (<i>Beta vulgaris</i>)	[103]	
	Stems	Wheat (<i>T. aestivum</i>)	[104]	
Phenol extraction (21)		Cork oak (<i>Quercus suber</i>)	[105]	Used in a large variety of tissues from various species, especially fruits, but often resulting in poor 2-DE profiling with few spots, and streaks, due to incomplete removal of interfering compounds
	Roots	<i>Pteris vittata</i>	[54]	
	Leaves	Tomato (<i>Solanum lycopersicum</i>)	[106]	
	Fruits	Strawberry (<i>Fragaria ananassa</i>)	[107]	
		Nectarine (<i>Prunus persica</i>)	[108]	
		Sweet orange (<i>Citrus sinensis</i>)	[109]	
	Seeds	Cacao (<i>Theobroma cacao</i>)	[110]	
	Stems	Kiwi (<i>Actinidia chinensis</i>)	[55]	
	Anthers	Maize (<i>Zea mays</i>)	[111]	
	Seeds	Cotton (<i>Gossypium arboreum</i>)	[112]	
TCA/acetone/phenol extraction (8)		Date palm (<i>Phoenix dactylifera</i>)	[68]	Effective in recalcitrant tissues, for example, fiber-rich tissues and fruits Maldonado et al. [72] reported that TCA/acetone/phenol extraction produced the best results among the three methods evaluated
	Fruits	Apricot (<i>Prunus armeniaca</i>)	[89]	
	Fiber	Cotton (<i>G. arboreum</i>)	[84]	
	Leaves	<i>A. thaliana</i>	[72]	
		Siberian spruce (<i>Picea obovata</i>)	[113]	
	Pollen	Holm oak (<i>Quercus ilex</i>)	[114]	

cold acetone. The air-dried protein pellet was dissolved in 2DE rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer, 20 mM DTT, and a trace amount of bromophenol blue).

- (5) 2DE and gel staining. This study aimed to identify the most significant changes in protein profiles between male and female plants; therefore, relatively insensitive CBB staining was used to visualize the proteins.

The results showed that the phenol-based protocol can effectively extract proteins from the powered tissues of *P. chinensis*, with protein yields of 5.4, 6.35, and 1.65 mg/g fresh weight for the leaf, stem phloem, and stem xylem, respectively. The protein yield from the stem xylem by phenol extraction was higher than that from stem xylem of the poplar tree in a previous report [116].

The 2DE analysis revealed a good resolution of most protein spots throughout the gel, with 193 ± 5 , 492 ± 10 and 360 ± 8 CBB-stained spots reproducibly detected, respectively, in stem xylem (Fig. 4), stem phloem, and leaf extracts [83]. Relatively fewer protein spots were detected in the xylem, in which metabolic activities are weak and

the majority of cells are dead. A total of ten protein spots were differentially expressed in the leaf and stem between both sexes, of which seven were successfully identified by MS analysis as NB-ARC domain containing protein, light harvesting chlorophyll a/b binding protein, ascorbate peroxidase, eukaryotic translation initiation factor 5A2, temperature-induced lipocalin, and phosphoglycerate kinase [83]. The sex-related difference was displayed in a tissue-specific way, especially in the stem. In particular, ascorbate peroxidase, temperature-induced lipocalin and phosphoglycerate kinase may be promising candidates to serve as protein molecular markers for sex determination in *P. chinensis*.

This example indicated that the general procedure for sample preparation in plant proteomics strongly depends on the species, the fragment being analyzed or even the stage of the plant development. Each cell type may contain a special protein population. Thus, the choice of plant material will determine the specific proteomic analysis. The protein extraction protocol to be used in each experiment needs to be chosen according to the plant material and the objectives of the specific research being conducted.

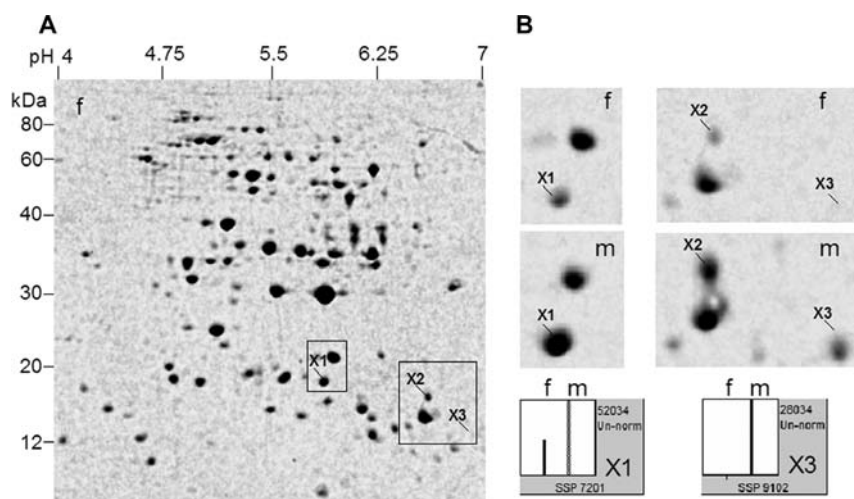


Figure 4. Identification of differentially expressed proteins in stems by 2DE between male and female plants in *Pistacia chinensis*. Stem was sampled in winter from 10-year-old plants. A mixed tissue powder from three different male or female individuals was used for protein extraction. (A) Protein profile of xylem proteins from female plants as reference. (B) Magnified gel regions containing spots X1–X3, accompanied by column configuration of relative abundance (generated by software PDQUEST). Spot X2 failed to be identified by MS/MS. f: female; m: male. The original picture was published in [83].

5 Current limitations and useful working limits

Over the past 10 years, TCA/acetone- and phenol-based methods have been applied for total protein extraction in plant proteomics. Because the techniques have been known for a very long time, the limits of these sample preparation techniques are also well known. For example, the major disadvantage of phenol extraction is that it is time-consuming.

A main limitation is that the protein precipitation and/or resolubilization steps involved in these techniques will result in protein losses. The key to good sample preparation is efficient protein solubilization with minimal handling [117]. However, the TCA/acetone- and phenol-based methods involve many wash steps, which is a problematic choice for most plant tissues. Carpentier et al. [13] clearly shows that protein precipitation is absolutely necessary when handling recalcitrant tissue. Thus, the loss of proteins is unavoidable, but the loss is reproducible. Despite this limitation, TCA/acetone precipitation and phenol extraction are useful as routine methods for recalcitrant plant tissues at present.

Another limitation is that in plant tissues, the TCA/acetone and phenol extraction methods often have to be optimized for each particular type of tissue and for the age of the organ [118] before selecting a fixed protocol because plants are highly differentiated organisms with many specialized organs and tissues. Thus, there is no single method of sample preparation that can be universally applied to all types of tissues analyzed by 2DE.

Different sample preparation protocols are complementary and useful in characterizing the whole plant proteome. For example, the comparison of a modified phenol-based protocol and a phenol-free protocol showed that the 2DE analysis of apple and strawberry fruit protein extracts revealed spots only present in phenol gels and other spots exclusive to the phenol-free samples [62].

6 Future developments

With the constant improvement of 2DE technology over the past three decades, especially the tremendous progress in plant proteomics over the last decade, sample preparation methods suitable for the 2DE analysis of plant tissues have been greatly improved. TCA/acetone- and phenol-based protocols are widely used for plant proteomic analyses.

Water stress has become one of the most critical abiotic factors affecting crop productivity at the global level. To better understand the mechanisms underlying the stress response in crops, proteomics-based projects are being increasingly utilized and will continue the discovery of novel gene targets for crop improvement [119]. These TCA/acetone- and phenol-based protocols are expected to still be very useful in total protein extraction in 2DE-based proteomics in the foreseeable future, especially in crop plant proteomics. However, compared with the model plant *Arabidopsis*, crops are more problematic for proteomic analyses mainly because their tissues contain large amounts of secondary compounds. Therefore, the described TCA/acetone- and phenol-based extraction methods need to be modified and refined when used for a specific crop tissue.

Moreover, these sample preparation methods can find many applications in other biochemical preparations, such as organelle isolation [120], sequential extraction [75], and gel-free proteomic analyses. These methods may be used with more sensitive separation and visualization methods, such as 2D DIGE [93]. This step is very important for projects in which many samples must be analyzed for comparative proteomic analysis. In addition, the continuous development of the new agents compatible with TCA, acetone, and phenol may be included in these methods for the optimal use.

Work in our laboratory was supported by the National Natural Science Foundation of China (grant no. 31371543) and partly by

the State Key Laboratory of Crop Biology (grant no. 2012KF01) at Shandong Agricultural University, China.

The authors have declared no conflict of interest.

7 References

- [1] Weiss, W., Görg, A., Sample solubilization buffers for two-dimensional electrophoresis. *Methods Mol. Biol.* 2008, **424**, 35–42.
- [2] Rabilloud, T., Lelong, C., Two-dimensional gel electrophoresis in proteomics: a tutorial. *J. Proteomics* 2011, **74**, 1829–1841.
- [3] Cottrell, J. S., Protein identification using MS/MS data. *J. Proteomics* 2011, **74**, 1842–1851.
- [4] Méchin, V., Damerval, C., Zivy, M., Total protein extraction with TCA–acetone. *Methods Mol. Biol.* 2007, **355**, 1–8.
- [5] Görg, A., Weiss, W., in: Rabilloud, T. (Ed.), *Proteome Research: Two-Dimensional Electrophoresis and Identification Methods*. Springer, Berlin, 2000, p. 57–106.
- [6] Damerval, C., Zivy, M., Granier, F., de Vienne, D., Two-dimensional electrophoresis in plant biology, in: Chrambach, A., Dunn, M., Radola, B. (Eds.), *Advances in Electrophoresis*. VCH, Weinheim, New York, 1988, pp. 265–340.
- [7] Shaw, M. M., Riederer, B. M., Sample preparation for two-dimensional gel electrophoresis. *Proteomics* 2003, **3**, 1408–1417.
- [8] Damerval, C., De Vienne, D., Zivy, M., Thiellement, H., Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat seedling proteins. *Electrophoresis* 1986, **7**, 52–54.
- [9] Granier, F., Extraction of plant proteins for two-dimensional electrophoresis. *Electrophoresis* 1988, **9**, 712–718.
- [10] Meyer, Y., Grosser, J., Chartier, Y., Cleyet-Marel, J. C., Preparation by two-dimensional electrophoresis of proteins for antibody production: antibodies against proteins whose synthesis is reduced by auxin in tobacco mesophyll protoplasts. *Electrophoresis* 1988, **9**, 704–712.
- [11] Rabilloud, T., Solubilization of proteins for electrophoretic analyses. *Electrophoresis* 1996, **17**, 813–829.
- [12] Wang, W., Scali, M., Vignani, R., Spadafora, A. et al., Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. *Electrophoresis* 2003, **24**, 2369–2375.
- [13] Carpentier, S. C., Witters, E., Laukens, K., Deckers, P. et al., Preparation of protein extracts from recalcitrant plant tissues: an evaluation of different methods for two-dimensional gel electrophoresis analysis. *Proteomics* 2005, **5**, 2497–2507.
- [14] Cho, K., Torres, N. L., Subramanyam, S., Deepak, S. N. et al., Protein extraction/solubilization protocol for monocot and dicot plant gel-based proteomics. *J. Plant Biol.* 2006, **49**, 413–420.
- [15] Isaacson, T., Damasceno, C. M., Saravanan, R. S., He, Y. et al., Sample extraction techniques for enhanced proteomic analysis of plant tissues. *Nat. Protoc.* 2006, **1**, 769–774.
- [16] Herbert, B. R., Righetti, P. G., Citterio, A., Boschetti, E., in: Wilkins, M. R., Appel, R. D., Williams, K. L., Hochstrasser, D. F. (Eds.), *Proteome Research: Concepts, Technology and Applications*, Springer, Berlin, Heidelberg, 2007, pp. 15–40.
- [17] Wu, X. L., Xiong, E. H., Wang, W., Scali, M., Cresti, M., Universal sample preparation method integrating trichloroacetic acid/acetone precipitation with phenol extraction for crop proteomic analysis. *Nat. Protoc.* 2014, doi:10.1038/nprot.2014.022.
- [18] O'Farrell, P. H., High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 1975, **250**, 4007–4021.
- [19] Hari, V., A method for the two-dimensional electrophoresis of leaf proteins. *Anal. Biochem.* 1981, **113**, 332–335.
- [20] Schuster, A., Davies, E., Ribonucleic acid and protein metabolism in pea epicotyls III. Response to auxin in aged tissue. *Plant Physiol.* 1983, **73**, 809–816.
- [21] Wu, F., Wang, M., Extraction of proteins for sodium dodecyl sulfate polyacrylamide gel electrophoresis from protease-rich plant tissues. *Anal. Biochem.* 1984, **139**, 100–103.
- [22] Cremer, F., Van de Walle, C., Methods for extraction of proteins from green plant tissues for two-dimensional polyacrylamide gel electrophoresis. *Anal. Biochem.* 1985, **147**, 22–26.
- [23] Flengsrud, R., Kobro, G., A method for two-dimensional electrophoresis of proteins from green plant tissues. *Anal. Biochem.* 1989, **177**, 33–36.
- [24] Hurkman, W. J., Tanaka, C. K., Solubilization of plant membrane proteins for analysis by two-dimensional electrophoresis. *Plant Physiol.* 1986, **81**, 802–806.
- [25] Grassman, W., Deffner, G., Verteilungschromatographisches Verhalten von Proteinen und Peptiden in phenolhaltigen Lösungsmitteln. *Hoppe-Seyler's Z. Physiol. Chem.* 1953, **293**, 89–98.
- [26] Görg, A., Weiss, W., Dunn, M. J., Current two-dimensional electrophoresis technology for proteomics. *Proteomics* 2004, **4**, 3665–3685.
- [27] Rose, J. K., Bashir, S., Giovannoni, J. J., Jahn, M. M., Saravanan, R. S., Tackling the plant proteome: practical approaches, hurdles and experimental tools. *Plant J.* 2004, **39**, 715–733.
- [28] Bodzon-Kulakowska, A., Bierzynska-Krzysik, A., Dylag, T., Drabik, A. et al., Methods for samples preparation in proteomic research. *J. Chromatogr. B* 2007, **849**, 1–31.
- [29] Wang, W., Tai, F. J., Chen, S. N., Optimizing protein extraction from plant tissues for enhanced proteomics analysis. *J. Sep. Sci.* 2008, **31**, 2032–2039.
- [30] Stalikas, C. D., Extraction, separation, and detection methods for phenolic acids and flavonoids. *J. Sep. Sci.* 2007, **30**, 3268–3295.
- [31] Croteau, R., Kutchan, T. M., Lewis, N. G., in: Buchanan, B., Gruissem, W., Jones, R. (Eds.) *Biochemistry and Molecular*

- Biology of Plants, American Society of Plant Physiologists, CRC Press, Rockville, MO, 2000, pp. 1250–1318.*
- [32] Vălcu, C. M., Schlink, K., Reduction of proteins during sample preparation and two-dimensional gel electrophoresis of woody plant samples. *Proteomics* 2006a, 6, 1599–1605.
 - [33] Wang, W., Vignani, R., Scali, M., Sensi, E. et al., Removal of lipid contaminants by organic solvents from oilseed protein extract prior to electrophoresis. *Anal. Biochem.* 2004, 329, 139–141.
 - [34] Toth, G. B., Pavia, H., Removal of dissolved brown algal phlorotannins using insoluble polyvinylpyrrolidone (PVPP). *J. Chem. Ecol.* 2001, 27, 1899–1910.
 - [35] Lerouxel, O., Cavalier, D. M., Liepman, A. H., Keegstra, K., Biosynthesis of plant cell wall polysaccharides—a complex process. *Curr. Opin. Plant Biol.* 2006, 9, 621–630.
 - [36] Vălcu, C. M., Schlink, K., Efficient extraction of proteins from woody plant samples for two-dimensional gel electrophoresis. *Proteomics* 2006b, 6, 4166–4176.
 - [37] Malik, N. S. A., Bradford, J. M., A simple protein extraction method for proteomic studies on olive leaves. *J. Food Agric. Environ.* 2005, 3, 246–248.
 - [38] Englard, S., Seifter, S., Precipitation techniques. *Methods Enzymol.* 1990, 182, 285–300.
 - [39] Giavalisco, P., Nordhoff, E., Lehrach, H., Gobom, J., Klose, J., Extraction of proteins from plant tissues for two-dimensional electrophoresis analysis. *Electrophoresis* 2003, 24, 207–216.
 - [40] Rodrigues, S. P., Ventura, J. A., Zingali, R. B., Fernandes, P. M. B., Evaluation of sample preparation methods for the analysis of papaya leaf proteins through two-dimensional gel electrophoresis. *Phytochem. Analysis* 2009, 20, 456–464.
 - [41] Nawrocki, A., Thorup-Kristensen, K., Jensen, O. N., Quantitative proteomics by 2DE and MALDI MS/MS uncover the effects of organic and conventional cropping methods on vegetable products. *J. Proteomics* 2011, 74, 2810–2825.
 - [42] Zadražnik, T., Hollung, K., Egge-Jacobsen, W., Meglič, V., Šuštar-Vozlič, J., Differential proteomic analysis of drought stress response in leaves of common bean (*Phaseolus vulgaris* L.). *J. Proteomics* 2013, 78, 254–272.
 - [43] Wang, W., Vignani, R., Scali, M., Cresti, M., A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. *Electrophoresis* 2006, 27, 2782–2786.
 - [44] Dogra, V., Ahuja, P. S., Sreenivasulu, Y., Change in protein content during seed germination of a high altitude plant *Podophyllum hexandrum* Royle. *J. Proteomics* 2013, 78, 26–38.
 - [45] Balbuena, T. S., Silveira, V., Junqueira, M., Dias, L. L. et al., Changes in the 2-DE protein profile during zygotic embryogenesis in the Brazilian pine (*Araucaria angustifolia*). *J. Proteomics* 2009, 72, 337–352.
 - [46] Link, A. J., LaBaer, J., Trichloroacetic acid (TCA) precipitation of proteins. *Cold Spring Harb. Protoc.* 2011, 2011, 993–994.
 - [47] Meisrimler, C. N., Lühje, S., IPG-strips versus off-gel fractionation: advantages and limits of two-dimensional PAGE in separation of microsomal fractions of frequently used plant species and tissues. *J. Proteomics* 2012, 75, 2550–2562.
 - [48] Saravanan, R. S., Rose, J. K., A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. *Proteomics* 2004, 4, 2522–2532.
 - [49] Vincent, D., Wheatley, M. D., Cramer, G. R., Optimization of protein extraction and solubilization for mature grape berry clusters. *Electrophoresis* 2006, 27, 1853–1865.
 - [50] Görg, A., Obermaier, C., Boguth, G., Csordas, A. et al., Very alkaline immobilized pH gradients for two-dimensional electrophoresis of ribosomal and nuclear proteins. *Electrophoresis* 1997, 18, 328–337.
 - [51] Chen, S., Harmon, A. C., Advances in plant proteomics. *Proteomics* 2006, 6, 5504–5516.
 - [52] Faurobert, M., Pelpoir, E., Chaib, J., Phenol extraction of proteins for proteomic studies of recalcitrant plant tissues. *Methods Mol. Biol.* 2007, 355, 9–14.
 - [53] Marsoni, M., Vannini, C., Campa, M., Cucchi, U. et al., Protein extraction from grape tissues by two-dimensional electrophoresis. *Vitis* 2005, 44, 181–186.
 - [54] Bona, E., Marsano, F., Massa, N., Cattaneo, C. et al., Proteomic analysis as a tool for investigating arsenic stress in *Pteris vittata* roots colonized or not by arbuscular mycorrhizal symbiosis. *J. Proteomics* 2011, 74, 1338–1350.
 - [55] Petriccione, M., Di Cecco, I., Arena, S., Scaloni, A., Scorticini, M., Proteomic changes in *Actinidia chinensis* shoot during systemic infection with a pandemic *Pseudomonas syringae* pv. *Actinidiae* strain. *J. Proteomics* 2013, 78, 461–476.
 - [56] Dupont, F. M., Tanaka, C. K., Hurkman, W. J., Separation and immunological characterization of membrane fractions from barley roots. *Plant Physiol.* 1988, 86, 717–724.
 - [57] Song, J., Braun, G., Bevis, E., Doncaster, K., A simple protocol for protein extraction of recalcitrant fruit tissues suitable for 2-DE and MS analysis. *Electrophoresis* 2006, 27, 3144–3151.
 - [58] Deytieux, C., Geny, L., Lapaillerie, D., Claverol, S. et al., Proteome analysis of grape skins during ripening. *J. Exp. Bot.* 2007, 58, 1851–1862.
 - [59] Negri, A. S., Prinsi, B., Scienza, A., Morgutti, S. et al., Analysis of grape berry cell wall proteome: a comparative evaluation of extraction methods. *J. Plant Physiol.* 2008, 165, 1379–1389.
 - [60] Vander Mijnsbrugge, K., Meyermans, H., van Montagu, M., Bauw, G., Boerjan, W., Wood formation in poplar: identification, characterization, and seasonal variation of xylem proteins. *Planta* 2000, 210, 589–598.
 - [61] Bayer, E. M., Bottrill, A. R., Walshaw, J., Vigouroux, M. et al., Arabidopsis cell wall proteome defined using multidimensional protein identification technology. *Proteomics* 2006, 6, 301–311.
 - [62] Zheng, Q., Song, J., Doncaster, K., Rowland, E., Byers, D. M., Qualitative and quantitative evaluation of protein

- extraction protocols for apple and strawberry fruit suitable for two dimensional electrophoresis and mass spectrometry analysis. *J. Agric. Food Chem.* 2007, **55**, 1663–1673.
- [63] Hu, X. L., Wu, X. L., Li, C. H., Lu, M. H. et al., Absciscic acid refines the synthesis of chloroplast proteins in maize (*Zea mays*) in response to drought and light. *PLoS ONE* 2012, **7**, e49500.
- [64] Briante, R., Patumi, M., Terenziani, S., Bismuto, E. et al., *Olea europaea*, L. leaf extract and derivatives: antioxidant properties. *J. Agric. Food Chem.* 2002, **50**, 4934–4940.
- [65] Röhrig, H., Schmidt, J., Colby, T., Bräutigam, A. et al., Desiccation of the resurrection plant *Craterostigma plantagineum* induces dynamic changes in protein phosphorylation. *Plant Cell Environ.* 2006, **29**, 1606–1617.
- [66] Gómez-Vidal, S., Tena, M., Lopez-Llorca, L. V., Salinas, J., Protein extraction from *Phoenix dactylifera* L. leaves, a recalcitrant material, for two-dimensional electrophoresis. *Electrophoresis* 2008, **29**, 448–456.
- [67] Dam, S., Laursen, B. S., Ornfelt, J. H., Jochimsen, B. et al., The proteome of seed development in the model legume *Lotus japonicus*. *Plant Physiol.* 2009, **149**, 1325–1340.
- [68] Sghaier-Hammami, B., Drira, N., Jorrín-Novo, J. V., Comparative 2-DE proteomic analysis of date palm (*Phoenix dactylifera* L.) somatic and zygotic embryos. *J. Proteomics* 2009, **73**, 161–177.
- [69] Aranjuelo, I., Molero, G., Erice, G., Avicé, J. C., Nogués, S., Plant physiology and proteomics reveals the leaf response to drought in alfalfa (*Medicago sativa* L.). *J. Exp. Bot.* 2011, **62**, 111–123.
- [70] Zhao, P. M., Wang, L. L., Han, L. B., Wang, J. et al., Proteomic identification of differentially expressed proteins in the Ligon lintless mutant of upland cotton (*Gossypium hirsutum* L.). *J. Proteome Res.* 2010, **9**, 1076–1087.
- [71] Madsen, E. B., Antolín-Llovera, M., Grossmann, C., Ye, J. et al., Autophosphorylation is essential for the *in vivo* function of the *Lotus japonicus* Nod factor receptor 1 and receptor-mediated signalling in cooperation with Nod factor receptor 5. *Plant J.* 2011, **65**, 404–417.
- [72] Maldonado, A. M., Echevarría-Zomeño, S., Jean-Baptiste, S., Hernández, M., Jorrín-Novo, J. V., Evaluation of three different protocols of protein extraction for *Arabidopsis thaliana* leaf proteome analysis by two-dimensional electrophoresis. *J. Proteomics* 2008, **71**, 461–472.
- [73] Jorrín, J. V., Maldonado, A. M., Castillejo, M. A., Plant proteome analysis: a 2006 update. *Proteomics* 2007, **7**, 2947–2962.
- [74] Wang, W., Wu, X. L., Xiong, E. H., Tai, F. J., Improving gel-based proteome analysis of soluble protein extracts by heat prefractionation. *Proteomics* 2012, **12**, 938–943.
- [75] Wu, X. L., Xiong, E. H., An, S. F., Gong, F. P., Wang, W., Sequential extraction results in improved proteome profiling of medicinal plant pinellia ternata tubers, which contain large amounts of high-abundance proteins. *PLoS ONE* 2012, **7**, e50497.
- [76] Rabilloud, T., Adessi, C., Giraudel, A., Lunardi, J., Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 1997, **18**, 307–316.
- [77] Galvani, M., Rovatti, L., Hamdan, M., Herbert, B., Righetti, P. G., Protein alkylation in the presence/absence of thiourea in proteome analysis: a matrix assisted laser desorption/ionization-time of flight-mass spectrometry investigation. *Electrophoresis* 2001, **22**, 2066–2074.
- [78] Castellanos-Serra, L., Paz-Lago, D., Inhibition of unwanted proteolysis during sample preparation: evaluation of its efficiency in challenge experiments. *Electrophoresis* 2002, **23**, 1745–1753.
- [79] Molloy, M. P., Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. *Anal. Biochem.* 2000, **280**, 1–10.
- [80] Herbert, B. R., Molloy, M. P., Gooley, A. A., Walsh, B. J. et al., Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. *Electrophoresis* 1998, **19**, 845–851.
- [81] Méchin, V., Consoli, L., Le Guilloux, M., Damerval, C., An efficient solubilization buffer for plant proteins focused in immobilized pH gradients. *Proteomics* 2003, **3**, 1299–1302.
- [82] Herbert, B., Advances in protein solubilisation for two-dimensional electrophoresis. *Electrophoresis* 1999, **20**, 660–663.
- [83] Xiong, E. H., Wu, X. L., Shi, J., Wang, X. Y., Wang, W., Proteomic identification of differentially expressed proteins between male and female plants in *Pistacia chinensis*. *PLoS ONE* 2013, **8**, e64276.
- [84] Li, Y. J., Zhang, X. Y., Wang, F. X., Yang, C. L. et al., A comparative proteomic analysis provides insights into pigment biosynthesis in brown colored fiber. *J. Proteomics* 2013, **78**, 374–388.
- [85] Prassinos, C., Rigas, S., Kizis, D., Vlahou, A., Hatzopoulos, P., Subtle proteome differences identified between post-dormant vegetative and floral peach buds. *J. Proteomics* 2011, **74**, 607–619.
- [86] Hossain, Z., Makino, T., Komatsu, S., Proteomic study of β -aminobutyric acid-mediated cadmium stress alleviation in soybean. *J. Proteomics* 2012, **75**, 4151–4164.
- [87] Sheoran, I. S., Ross, A. R., Olson, D. J., Sawhney, V. K., Differential expression of proteins in the wild type and 7B-1 male-sterile mutant anthers of tomato (*Solanum lycopersicum*): a proteomic analysis. *J. Proteomics* 2009, **71**, 624–636.
- [88] Zhang, D., Ren, L., Yue, J. H., Wang, L. et al., A comprehensive analysis of flowering transition in *Agapanthus praecox* ssp. *orientalis* (Leighton) Leighton by using transcriptomic and proteomic techniques. *J. Proteomics* 2013, **80**, 1–25.
- [89] D'Ambrosio, C., Arena, S., Rocco, M., Verrillo, F. et al., Proteomic analysis of apricot fruit during ripening. *J. Proteomics* 2013, **78**, 39–57.
- [90] Vincent, D., Ergül, A., Bohlman, M. C., Tattersall, E. A. R. et al., Proteomic analysis reveals differences between *Vitis vinifera* L. cv. Chardonnay and cv. Cabernet Sauvignon and their responses to water deficit and salinity. *J. Exp. Bot.* 2007, **58**, 1873–1892.

- [91] Rossignol, M., Analysis of the plant proteome. *Curr. Opin. Biotech.* 2001, 12, 131–134.
- [92] Agrawal, G. K., Sarkar, A., Righetti, P. G., Pedreschi, R. et al., A decade of plant proteomics and mass spectrometry: translation of technical advancements to food security and safety issues. *Mass Spectrom. Rev.* 2013, 32, 335–365.
- [93] Gao, L. Y., Wang, A. L., Li, X. H., Dong, K. et al., Wheat quality related differential expressions of albumins and globulins revealed by two-dimensional difference gel electrophoresis (2-D DIGE). *J. Proteomics* 2009, 73, 279–296.
- [94] Islam, M. A., Sturrock, R. N., Ekramoddoullah, A. K., A proteomics approach to identify proteins differentially expressed in Douglas-fir seedlings infected by *Phellinus sulphurascens*. *J. Proteomics* 2008, 71, 425–438.
- [95] Thomas, L., Marondedze, C., Ederli, L., Pasqualini, S., Gehring, C., Proteomic signatures implicate cAMP in light and temperature responses in *Arabidopsis thaliana*. *J. Proteomics* 2013, 83, 47–59.
- [96] Miranda, H., Cheregi, O., Netotea, S., Hvidsten, T. R. et al., Co-expression analysis, proteomic and metabolomic study on the impact of a Deg/HtrA protease triple mutant in *Synechocystis* sp. PCC 6803 exposed to temperature and high light stress. *J. Proteomics* 2013, 78, 294–311.
- [97] Wang, C. Y., Shen, R. F., Wang, C., Wang, W., Root protein profile changes induced by Al exposure in two rice cultivars differing in Al tolerance. *J. Proteomics* 2013, 78, 281–293.
- [98] Salavati, A., Khatoon, A., Nanjo, Y., Komatsu, S., Analysis of proteomic changes in roots of soybean seedlings during recovery after flooding. *J. Proteomics* 2012, 75, 878–893.
- [99] Kieffer, P., Schröder, P., Dommès, J., Hoffmann, L. et al., Proteomic and enzymatic response of poplar to cadmium stress. *J. Proteomics* 2009, 72, 379–396.
- [100] Chernov, V. M., Chernova, O. A., Medvedeva, E. S., Mouzykantov, A. A. et al., Unadapted and adapted to starvation *Acholeplasma laidlawii* cells induce different responses of *Oryza sativa*, as determined by proteome analysis. *J. Proteomics* 2011, 74, 2920–2936.
- [101] Immel, F., Renaut, J., Masfaraud, J. F., Physiological response and differential leaf proteome pattern in the European invasive Asteraceae *Solidago canadensis* colonizing a former cokery soil. *J. Proteomics* 2012, 75, 1129–1143.
- [102] Wang, L., Ma, H., Song, L., Shu, Y., Gu, W., Comparative proteomics analysis reveals the mechanism of pre-harvest seed deterioration of soybean under high temperature and humidity stress. *J. Proteomics* 2012, 75, 2109–2127.
- [103] Li, H., Cao, H., Wang, Y., Pang, Q. et al., Proteomic analysis of sugar beet apomictic monosomic addition line M14. *J. Proteomics* 2009, 73(2), 297–308.
- [104] Bazargani, M. M., Sarhadi, E., Bushehri, A. A., Matros, A. et al., A proteomics view on the role of drought-induced senescence and oxidative stress defense in enhanced stem reserves remobilization in wheat. *J. Proteomics* 2011, 74, 1959–1973.
- [105] Ricardo, C. P., Martins, I., Francisco, R., Sergeant, K. et al., Proteins associated with cork formation in *Quercus suber* L. stem tissues. *J. Proteomics* 2011, 74, 1266–1278.
- [106] Rocco, M., Corrado, G., Arena, S., D'Ambrosio, C. et al., The expression of tomato prosystemin gene in tobacco plants highly affects host proteomic repertoire. *J. Proteomics* 2008, 71, 176–185.
- [107] Bianco, L., Lopez, L., Scalone, A. G., Di Carli, M. et al., Strawberry proteome characterization and its regulation during fruit ripening and in different genotypes. *J. Proteomics* 2009, 72, 586–607.
- [108] Giraldo, E., Díaz, A., Corral, J. M., García, A., Applicability of 2-DE to assess differences in the protein profile between cold storage and not cold storage in nectarine fruits. *J. Proteomics* 2012, 75, 5774–5782.
- [109] Pan, Z., Zeng, Y., An, J., Ye, J. et al., An integrative analysis of transcriptome and proteome provides new insights into carotenoid biosynthesis and regulation in sweet orange fruits. *J. Proteomics* 2012, 75, 2670–2684.
- [110] Noah, A. M., Niemenak, N., Sunderhaus, S., Haase, C. et al., Comparative proteomic analysis of early somatic and zygotic embryogenesis in *Theobroma cacao* L. *J. Proteomics* 2013, 78, 123–133.
- [111] Uváčková, L., Takáč, T., Boehm, N., Obert, B., Samaj, J., Proteomic and biochemical analysis of maize anthers after cold pretreatment and induction of androgenesis reveals an important role of anti-oxidative enzymes. *J. Proteomics* 2012, 75, 1886–1894.
- [112] Du, S. J., Dong, C. J., Zhang, B., Lai, T. F. et al., Comparative proteomic analysis reveals differentially expressed proteins correlated with fuzz fiber initiation in diploid cotton (*Gossypium arboreum* L.). *J. Proteomics* 2013, 82, 113–129.
- [113] Kjellsen, T. D., Shiryaeva, L., Schröder, W. P., Strimbeck, G. R., Proteomics of extreme freezing tolerance in Siberian spruce (*Picea obovata*). *J. Proteomics* 2010, 73, 965–975.
- [114] Valero, G. J., Valledor, L., González, F. R., Navarro Cerrillo, R. M., Jorrín-Novo, J. V., Proteomic analysis of Holm oak (*Quercus ilex* subsp. *ballota* [Desf.] Samp.) pollen. *J. Proteomics* 2012, 75, 2736–2744.
- [115] Wang, X. R., Liu, W. Z., Development of oil bodies in the fruit of *Pistacia chinensis*. *Chin. Bullet Bot.* 2011, 46, 665–674.
- [116] Christensen, J. H., Bauw, G., Welinder, K. G., Van Montagu, M., Boerjan, W., Purification and characterization of peroxidases correlated with lignification in poplar xylem. *Plant Physiol.* 1998, 118, 125–135.
- [117] Wilkins, M. R., Sanchez, J. C., Williams, K. L., Hochstrasser, D. F., Current challenges and future applications for protein maps and post-translational vector maps in proteome projects. *Electrophoresis* 1996, 17, 830–838.
- [118] Islam, N., Lonsdale, M., Upadhyaya, N. M., Higgins, T. J. et al., Protein extraction from mature rice leaves for two-dimensional gel electrophoresis and its application in proteome analysis. *Proteomics* 2004, 4, 1903–1908.
- [119] Roya, A., Rushton, P. J., Rohila, J. S., The potential of proteomics technologies for crop improvement under drought conditions. *Crit. Rev. Plant Sci.* 2011, 30, 471–490.
- [120] Tai, F. J., Yuan, Z. L., Wu, X. L., Zhao, P. F. et al., Identification of membrane proteins in maize leaves, altered in stress through polyethylene glycol treatment. *Plant Omics J.* 2011, 4, 250–256.