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Leaf proteome analysis of wheat-rye translocation lines

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Abstract

Wheat-rye translocation lines were developed to produce a main crop resistant to biological and physical stress. Parental line of rye cv. "Chaupon" contains 2RL chromatin to harbor resistance genes for powdery mildew and leaf rust. In order to 2RL-derived rye proteins and 2RL-perbuted proteins in wheat-rye translocation lines, the gel-based proteomics employed with "Coker 797" (non-2RL) and near isogenic line (stabilized 2RL).The leaf proteome was resolved on 2D-gel, and 216 spots were selected for mass spectrometry analysis by image analyzer. A total of 90 proteins with isoform (65 non-reductant proteins) were identified with the identification success rate of 42% using MALDI MS or MS/MS. The identified proteins were classified by functional annotation based on biological processes: carbohydrate metabolic process (11%), cellular amino acid metabolic process (6%), DNA integration / replication (3%), lipid metabolic process (3%), photosynthesis (23%), protein modification / biosynthesis (11%), response to stimulus (8%), transcription and translation (6%), transport (9%), unknown (12%), other biological processes (8%). A total of 24 proteins were differentially expressed between non-2RL and 2RL lines, in which β-glucosidase, in particular, originated from the chromosome 2RL of rye, was exclusively appeared in NIL. In addition, small Ras-related GTP binding-protein assigned to wheat was predominantly found in 2RL rye chromatin-possessing NIL. These results suggested that the acquired genetic traits obtained from rye 2RL enhance the resistance to biotic and abiotic stress such as Dehydration-induced protein; heat shock protein (HSP 26.6 and 26.6B) in wheat-rye translocation lines by altered the proteome expression.

Keywords: 2RL-NIL, non-2RL "Croker", Proteomics, Wheat-rye translocation line.
Abbreviations: 2DE two-dimensional electrophoresis, MALDI matrix assisted laser desorption/ionization, MS mass spectrometry, NIL near isogenic line, RL recombinant line, TOF time-of-flight.

Introduction

Wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD) grain is the principal cereal as the second most produced food among the whole cereal crop. Cultivated rye (*Secale cereale* L., 2n=2x=14, RR) is one of the major cereal crops for both grain and forage. The tolerance of rye is higher to dryness and chilling stress than that of wheat. In addition, rye has been known to be more resistant to the pest than wheat. Thus, several molecular breeding has been attempted through the introgression of rye chromosome arms to wheat chromosome for the consistently resistant wheat-rye translocation lines. Recently the yield potential of wheat grain has been increased by acquiring traits for the biotic and abiotic tolerance (Rajaram et al., 2008). It is expected that agricultural biotechnology require an annual yield of about 2% until 2020 to meet future demands for worldwide wheat (Reynolds et al., 2008). However, the natural wild type wheat cultivar is more susceptible to the pest *Fusarium*-causing disease, resulting in the notoriously low production (Ban and Watanabe, 2001). The Environmental Protection Agency stated that the pesticides in current use should be strictly regulated since the chemical pesticides give the severe risk to the human and ecological system (Shurdut et al., 1998). Thus, the need and study of natural tolerance and resistance to biotic and abiotic stress will be increased through the evaluation and elucidation of biochemical, molecular and biological mechanisms in wheat species. Rye is an important source of alien genes because of tolerance to biotic and abiotic stresses (Mater et al., 2004). The breeding of wheat-rye hybrid has been developed toward the increase of useful genetic variation of wheat genome and the decrease of the traits of unwanted rye chromatin (Pena et al., 1990; Kim et al., 2004). Among the several molecular breeding attempts, the target rye chromatin 1R (1RS.1AL, 1RS.1BL, 1RS.1DL) and 2R (2AS.2RL, 2RS.2BL, 2BS.2RL) have been replaced with the corresponding chromatin of wheat (Ehdaie et al., 2003; Hysing et al., 2007). The most common translocation

line is the 1BL/1RS. Far from the merits of pest-resistant rye 1R short-chromosome, the wheat-rye translocation contains still several problems such as reduced gluten strength, dough stickiness, and poor loaf volume of wheat flour (Fenn et al., 1994). Another line, 'Hamlet' (PI549276) is designed for the strengthened resistance trait against Hessian fly (*Mayetiola destructor* Say) larvae (Friebe et al., 1990). The Hamlet-line was obtained by crossing between wheat cultivar ND7532 and rye cultivar 'Chaupon' to translocate the chromosome 2RL (*H21* resistant gene to biotype L of Hessian fly) from rye to wheat (Jang et al., 1999). Thus, 2BS.2RL wheat-rye translocation line acquired the resistance for powdery mildew and leaf rust (Sears et al., 1992). Seo et al., (2001) developed near-isogenic lines (NILs) for 2RL through continuous selection of NILs by back-crossing non 2RL-Coker797 and 2RL-Hamlet as a recurrent parent wheat cultivar and a donor parentfor the field application, respectively.In previous studies, to elucidate the molecular interaction between wheat-rye translocation line (2BS.2RL) and the Hessian fly, cDNA library from NILs with *H21* infested by larvae of Hessian fly. By the sequencing of ESTs, the partial sequences of 730 ESTs were obtained and registered in the Gene Bank (Jang et al., 2003). However, the proteomics of 2BS.2RL translocation lines has not been reported except for the recent proteomics of 1BL.1RS translocation on the wheat endosperm (Gobaa et al., 2007). The rapidly developing technique, recent proteomics is becoming a powerful tool to investigate genome-wide protein expression involved in the specific function and metabolism of model plants (Pandey and Mann, 2000). 2-DE based proteomic approach is a simple and powerful method for separating complex protein mixtures into single protein by its own molecular weight (M*r*) and isoelectric point (p*I*). However, the resolutions of protein spots were restricted to the fully solubilized proteins on 2D-gel (Kamal et al., 2010a). When 2DE sample contains the insoluble proteins and non-proteinous compounds such as carbohydrates, lipids, nucleic acids and phenolic compounds, these may interfere with the separation of the proteins or their subsequent visualization. Also, terpenoids, pigments and wax-like lipid polymers produce spot streaking and charge heterogeneity on 2D-gel. Thus, the protein extraction and solubilization step is the most critical for high resolution of 2DE. The wheat tissues include plenty of carbohydrates and polymeric starch produced by photosynthesis and condensation of carbohydrates, which is in turn repositioning from source organ (leaf). The present study was focused on the proteomic profiles of leaf from 2BS.2RL wheat-rye translocation line. In addition, the 2D gel-based comparative proteomics between 2RL-Coker797 and 2RL-translocating Near Isogenic Line (NIL) was employed to identify the constitutively expressed proteins related to 2RL resistance to biotic and abiotic stresses.

Results

Identification of the leaf proteins from wheat-rye translocation lines

In order to remove the interfering contaminant, several extraction methods were applied to the wheat tissues in the present study. The protein extraction methods include deionized water precipitation, direct rehydration buffer precipitation and TCA/acetone precipitation. In the preliminary experiments, deionized water extraction and subsequent rehydration with urea/thiourea/CHAPS resulted in exclusive separation of acidic proteins. Direct application of rehydration buffer with medium stringent chaotrope made no good result, however, the TCA/acetone precipitation method proved good resolution of proteins with broad ranges of M*r* and p*I* (data not shown). Thus, the optimal extraction method was chosen as the standard protocol for the high-quality separation of wheat proteins using 8 M urea/2% CHAPS/20% TCA. To better understand the physiology and biochemistry of wheat-rye translocation lines and to select the agronomically important wheat varieties, the proteomic approaches were employed to display the expressed leaf proteome of two lines. In general, the growing wheat leaves undergo actively photosynthesis and distribute the carbon source dynamically to sink organs such as stem and root. The representative proteomic 2D-gel images of leaf of NIL were shown in Fig. 1. When the wheat proteomes were displayed in 3-10 NL IPG gel, the majority of proteins were distributed in the acidic region of pH 3.5-5 (Fig. 1). When the automatic spot detection was performed by the image analysis program Progenesis Workstation ver. 2005, approximately 1100-1500 protein spots were detected. The numbers of MS-identifiable spots suggest being around 100-150. In order to map the reference 2D-gel of wheat leaf proteome, all available protein spots were collected and subjected to allow MS analysis. In total, 216 protein spots were chosen and followed by in-gel tryptic digestion, MS/MS analysis and Mascot search against wheat/rye, rice, *Arabidopsis* and other plant genomic database in a sequential manner. Out of 216 spots, 90 spots were identified by home-made database search with the identification success rate of 42%. Proteins identified by wheat (*Triticum aestivum*) and rye (*Secale cereale*) NCBI database were counted for 38 (42%)) and 4 (4%) calculated by identified 90 spots, respectively. The remaining spots were in majority hit by rice (*Oryza sativa*, 37 hit) and others by other plant databases (*Arabidopsis thaliana, Hordeun vulgare, Physcomitrella patens, Zea mays, Solanum lycopersicum, Oryza tauri, Triticum turgidum).* From the reference wheat leaf 2D-gel, the most abundant protein spots corresponding to 53 kDa within the broad horizontal p*I* range revealed ribulose 1,5-bis-phosphate carboxylase/oxygenase large subunit (RbcL, spot #59, 63, 84, 98, 102, 118, 213). The second most abundant protein corresponding to 19 kDa revealed ribulose 1,5-bis-phosphate carboxylase/oxygenase small subunit (RbcS, spot #195, 196, 198, 199, 201). The multi-protein spots assigned as the same protein are supposed to be caused by the heterogeneity of post-translational modification. The identified proteins were classified by functional annotation according to the classification criterion (Huang et al., 2003), which is involved in carbohydrate metabolic process (11%), cellular amino acid metabolic process (6%), DNA integration / replication (3%), lipid metabolic process (3%), photosynthesis (23%), protein modification / biosynthesis (11%), response to stimulus (8%), transcription and translation (6%), transport (9%), unknown (12%), other biological processes (8%) (Fig. 2). In particular, it is noted that several stress-induced proteins (dehydration-induced protein, spot #9; small heat shock protein, spot #100, #113; chitin-inducible gibberellin-responsive protein, spot #186) and regulatory proteins (Myb transcription factor, spot #74; small Ras-related GTP-binding protein, spot #151; POZ domain protein, spot #172, 191) were found in the wheat leaf proteomic analysis.

Comparison of leaf proteome expression profile between wheat and wheat-rye translocation line

As shown in Fig. 2, the most of wheat leaf proteins were found to be involved in carbohydrate metabolism. In general, plant plays a critical role to balance the carbon partition

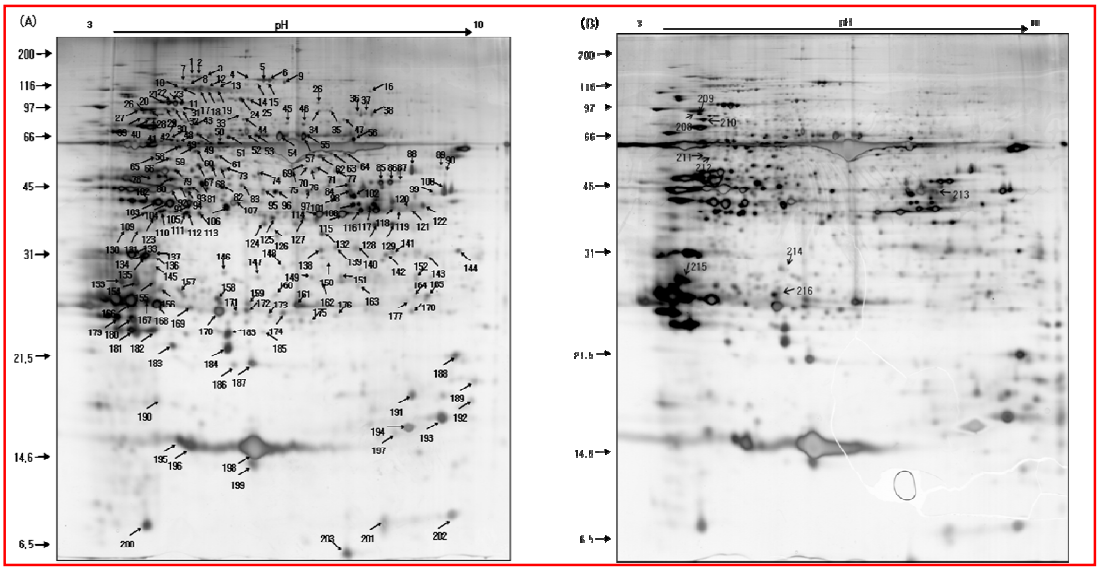

Fig 1. Reference 2-DE gel map from the wheat-rye translocation line leaf proteome of (A) NIL, (B) Coker797. Proteins were extracted from leaf, separated by 2-DE, and visualized by silver staining. The MW of each protein was determined by 2-DE markers.

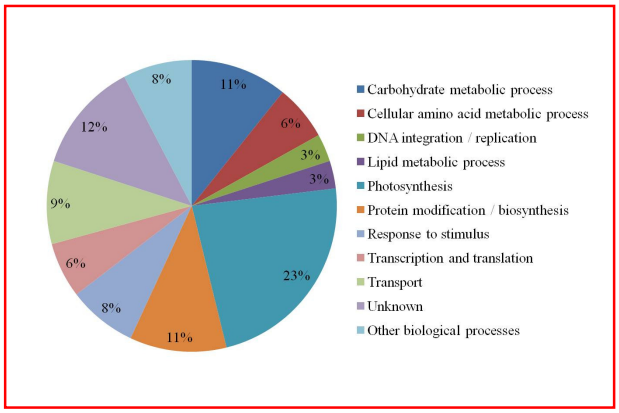

Fig 2. Frequencies distribution of identified proteins within functional categories based on biological processes. Classifications were made using *i*ProClass and NCBI (BLASTtx) databases, and assignment by function was based on gene ontology (see Supplementary Table 1)

between source organ (leaf) and sink organ (root). As a source organ, the leaves incessantly fix the carbon dioxide by the electrons and reducing powers synthesized by light reaction and relocate various types of carbohydrates to the sink source via stem tissue. Thus, the leaves should have the complicated carbon metabolic enzymes and act their catabolic or anabolic reactions through concerted coordination. Total 12 proteins were assigned as glycolysis, sucrose and starch metabolism. Interestingly, seven and five proteins were assigned as Rubisco large and small subunit, respectively. In order to investigate the change of leaf proteome between non 2RL-Coker797 and 2RL-NIL, the soluble leaf proteins were separated on 2D-gel and stained with silver staining, and further comparative image analysis was performed. Based on the reference 2D gel map of wheat leaf, the local zones of 2D-gels were magnified and compared in detail whether the specific expression patterns were changed (Fig. 3). As listed in Supplementary Table 1, 24 proteins spots were accounted for the differential expressed proteins, of which proteins could be grouped into four groups. Group A proteins belonged to the up-regulated proteins in NIL compared to Coker797, while Group B proteins belonged to the down-regulated ones. Group C and D corresponded to the proteins appeared and disappeared exclusively in NIL, suggesting to be caused by either direct expression of 2RL-rye chromosome or indirectly induced wheat gene by introduced 2RL (Fig. 3).

Specific protein analysis of wheat-rye translocation lines

As shown in Figure 3, as an example of the up-regulated (spot # 40) and down-regulated (spot # 28, 41) protein in NIL, which was as cell division protease ftsH homolog 2, chloroplast precursor annotated from rice (*Oryza sativa*), that are unevenly affected in our study due to post translational modification of proteins. Furthermore, transposon protein (spot # 176) transparently up-regulated in NIL that is proteolysis into smaller polypeptides by cleavage of their peptide bonds, and POZ domain protein (spot # 191) act as protein binding. In our study, RbcL, RbcS, ATP synthase

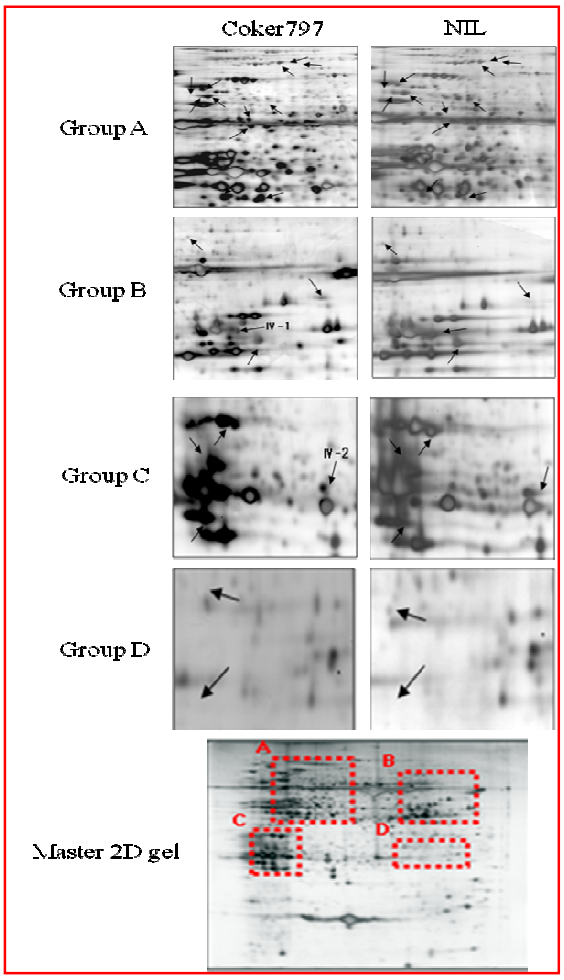

Fig 3. Differential proteins expression between Coker797 and Near-isogenic line in wheat leaf. From the master 2D-gel image (bottom), four group representing zone A, B, C and D were magnified and displayed between Coker797 and NIL.

beta subunit and geranylgeranyl hydrogenase proteins were down-regulated; those are involved in photosynthesis couple to carbon metabolism. The proteins belonged to Group C, which were exclusively expressed in NIL wheat, were appeared likely to hypothetical proteins, Rubisco large subunit and retrotransposon protein. Interestingly, spot 43 was exclusively identified as β-glucosidase from rye (*Secalae cereale*, 2RL chromosome). MS/MS spectrum of the tryptic digest GIDYYNNLINSLIR was significantly assigned as β-glucosidase of rye. The enzyme β-glucosidase converts glucoside to α-glucose or trans-converts 1, 4-β-glucan to β-glucose, vice versa. This enzyme was also mapped on sucrose metabolism as depicted as Figure 2. By the multiple amino acid sequence analysis, the sequence of ScGlu1 (β-glucosidase from *Secalae cereale*) resembled so much with that of TaGlu1-3 (β-glucosidase isoform I, 2, 3 from *Triticum aestivum*) with sequence homology of 95%. It is noted that the predicted peptide size of the tryptic peptide #2 originated from rye was shorter than that of tryptic peptide from wheat. Thus, it supports strongly that the MS/MS spectrum of spot #43 is correctly fragmented from the rye source. Other interesting protein belonged to Group C is spot #151, of which tryptic peptide FYCWDTAGQK was assigned by peptide fragmentation of *b*- and *y*-ion series from small Ras-related GTP-binding protein of *Triticum aestivum*. To the recent knowledge, the Ras-related G protein found in wheat was the first case, of which the protein was exclusively expressed by the introduction of rye chromosome. The acyl-[acyl-carrier-protein] desaturase,chloroplast precursor proteins belonged to Group D, which were disappeared in NIL.

Discussion

Characteristics of wheat leaf proteome (T. aestivum L.)

Wheat grain is a fundamental source of human major food, however, the wheat cultivars in nature are vulnerable to the diseases and environmental changes. Thus, the molecular breeding for high yields and acquired resistance has been tried with the translocation of rye chromosome containing resistance genes into the wheat chromosome (Rabinovich, 1998). As an initial attempt of wheat molecular breeding, the introduction to the short arm of rye chromosome 1R showed the positive effects on the yield, stress tolerance and adaptation of wheat (Merker et al., 1982). Later, this translocation line was turned out to have detrimental effect of reduced bread-making quality due to the absence of wheat chromosome arm 1BS (Martin and Stewart, 1986). In contrast to 1R, the presence of 2R in wheat improved to produce the content of kernel arabinoxylans essential for both baking and nutritional quality of cereals (Vinkx and Delcour, 1996). In addition to the enhanced wheat grain, 2RL has known to contain genes resistant to powdery mildew (Heun and Friebe, 1990), leaf and stem rust (Brunell et al., 1999) and Hessian fly (Jang et al., 1999). Thus, the present study was focused to characterize the proteomic property of 2BS.2RL wheat-rye translocation. Furthermore, this study aimed to map and catalogue the leaf proteome and to compare the leaf proteome of non-2RL and 2RL-translocated wheat lines. So far, several proteomic analysis of wheat has been conducted to evaluate the wheat cultivars and varieties. Proteomic analysis of wheat amyloplasts was attempted to understand the physiological and biochemical properties during grain-filling process (Andon et al., 2002). In additions, the proteomic analyses were conducted to determine the protein expression of chromosome-deleted wheat seed and the differential expression profiles among diploid, tetraploid and hexaploid wheat flour (Islam et al., 2003a; 2003b). The aphid pest-induced wheat leaf proteome was attempted to find the molecular markers resistant to biotic stress (Porter and Webster, 2000). Recently, the global 2D-based wheat leaf proteome (Donnelly et al., 2005) and the proteomic analysis of 1BL.1RS translocation wheat endosperm (Gobaa et al., 2007) were reported. However, the proteomic attempt of 2BS.2RL translocation has not been studied yet. A representative leaf proteome map of 2RL-wheat line was constructed as shown in Figure 1. The most abundant soluble proteins, ribulose 1,5-bis-phosphate carboxylase/oxygenase large (RbsL, 53 kDa, p*I* 6.2) and small (RbsS, 19 kDa, p*I* 8.8) subunits were clearly observed in leaf. The accumulation of Rubisco, a major Calvin-cycle enzyme, is known to be occupied with approximately half amount of total leaf proteins (Schiltz et al., 2004). The identified total proteins, 90 out of 216, the initial trial, were identified by Mascot search against wheat/rye, rice and other plant NCBI genomic databases with the success rate of 42%, which is comparable to the previous wheat leaf proteome study of that of 51% (Donnelly et al., 2005). As shown in Figure 1 and Supplementary Table 1, many isofoms of proteins and isoproteins were detected as previously

described (Koller et al., 2002; Sheffield et al., 2006). A number of proteins with multiple isoforms and isoproteins were observed from metabolic enzymes such as lipoxygenase, cell division protease, ATP synthase F1 subunit α subunit, RubisCO large/small subunit, including hypothetical protein and POZ domain protein. It is suggested that the multiple spots could be originated from the protein heterogeneity with different signal or target sequences, the post-translational modifications, diverse protein degradation and carbamylation or overabundant streaking of RubisCO on 2D-gel (Donnelly et al., 2005). The functional categorization of identified 90 proteins (65 unique proteins) could be classified to the metabolism (20%) as shown in Figure 2. Furthermore, these proteins can be subdivided into carbohydrate metabolism (11%) with a majority of group, where metabolic enzymes showed great redundancy. However, the property of functional classification of the present proteomic study showed different result compared to the previous leaf proteome done by Donnelly's group. Probably, it may be caused by the different extraction method and database search algorithm. Most of proteins were involved in energy and carbohydrate metabolism as would be expected in plant leaf tissue (Watson et al., 2003). The proteins involved in carbohydrate metabolism included glycolysis, sucrose/starch metabolism and carbon fixation as mapped on metabolic pathway. This metabolism related proteins with the proteomic study helps to give the insight of the physiological and biochemical process in the plant tissue (Dupont, 2008). Since the leaf tissue is considered carbon source, most of leaf proteins play a key role of active carbohydrate synthesis and repositioning carbon source as sucrose form to carbon sink organ via stem (Lalonde et al., 2004).

Implication of differential proteome expression in wheat-rye translocation line

This study was designed to investigate the change of leaf proteome between non 2RL-Coker797 and 2RL-NIL and to identify the marker proteins induced by the introduction of 2RL. Based on the reference 2D gel map of NIL wheat leaf, the differentially expressed proteins were compared to non-2RL wheat leaf and listed as catalogued in Supplementary Table 1. In total, 24 proteins were engaged in the 2RL-induced proteins, of which proteins can be grouped into four groups: up-regulated, down-regulated, exclusively appeared and disappeared proteins in 2RL-NIL. Of the 24 proteins identified differentially expressed proteins, 19 proteins are involved in sorts of metabolic process, photosynthesis, transports and so on, while the remaining 5 proteins was annotated as hypothetical proteins due to a lack of known sequence homology. Interestingly, wheat RubisCO large subunit revealed as down-regulated and appeared exclusively in NIL while wheat RubisCO small subunit as disappeared. In a previous study on the salt-induced wheat leaf proteome, salt stress induced RubisCO large subunit and repressed RubisCO small subunit (Caruso et al., 2008). Thus, the altered expression of RubisCO in 2RL-wheat leaf may give an indication of abiotic stress. As shown in Figure 3, β-glucosidase was identified and mapped on sucrose metabolism, in which β-glucosidase catalyzes the hydrolytic cleavage of 1,3-β glucosidic linkages and trans-converts 1,4-β-glucan to β-glucose or vice versa (Caruso et al., 2008). Interestingly, β-glucosidase was exclusively appeared in 2RL-possessing NIL, and furthermore, the protein was originated from rye. Thus, the finding of rye β-glucosidase supports strongly the acquired trait from rye 2RL chromosome to wheat chromosome. By the comparison of β-glucosidase between rye and wheat, rye β-glucosidase showed characteristic pattern of peptide mass fingerprinting, i.e. the terminal amino acid residue of second peptide from rye was different from that of wheat. The enzyme, the presence of β-glucosidase includes the following biological implications. First, it is involved in the alteration of specific β-linked polysaccharides during cell expansion in development (Leah et al., 1995). Second, it is involved in pathogen defence reaction by cyanogenesis by the hydrolytic catalysis of glucosides after pathogen's attack (Hughes et al., 1992). Third, it is involved in the release of active plant hormone such as cytokinin, gibberellin and auxin from the biologically inactive hormone-glucoside conjugate (Brzobohatý et al., 1993). Another interesting protein found exclusively in 2RL-NIL was small Ras-related GTP-binding protein as shown in Figure 3. Plant Ras-related G protein is known to regulate diverse cellular function and signal transduction. (Holstein et al., 2003). To my best knowledge, the Ras-related G protein found in wheat was the first case in this study, since the identification of Ras-related G protein was first found in *Arabidopsis thaliana* previously (Matsui et al., 1989). The appearance of wheat regulatory G-protein may be caused by the introduction of 2RL chromosome, resulting in the perturbation of wheat proteome as observed ESTs of wheat-rye translocation (Jang et al., 1999). Other small GTP protein-binding protein, called RanBP, was isolated from a wheat cDNA library, in which RanBP was supposed to function in nucleocytoplasmic transport and mitosis (Tian et al., 2006). The present proteomic study on wheat-rye translocation line can provide the useful information on the understanding of biological markers for biotic and abiotic resistance due to the acquired traits from 2RL rye chromosome. Additionally, acyl-[acyl-carrier-protein] desaturase,chloroplast precursor proteins disappeared in NIL, which was introduce double bonds into fatty acids that are bound to ACP (acyl carrier protein); they are present in the stroma of plant plastids (McKeon and Stumpf, 1992), endoplasmic reticulum, the chloroplast membrane in plant cells (Jaworski, 1987), and the thylakoid membrane in cyanobacterial cells (Wada et al., 1993). Such type of desaturase is the most proficient regulator of the extent of un-saturation of membrane lipids in response to amends in temperature. Acyl-lipid desaturases has been play important role in the tolerance and acclimatization to cold of cyanobacteria, resulting to the genetic manipulation of desaturases in relation to adaptation of cold tolerance, and the temperature-regulated expression of genes for desaturases (Murata and Wada, 1995).

Materials and methods

Plant Materials

The two lines (1RL-Coker797 and 2RL-NIL) made by back cross introgession (Jang et al., (1999, 2003)), which were further grown in the field for proteomics analysis.

Preparation of plant proteins

For proteomic analysis, the flag leaves were cut at 7 days after anthesis, and quickly frozen in deep freezer prior to use. The tissue of each leaf was pulverized in liquid nitrogen, and the powder was suspended in sample buffer I (0.3% (w/v) SDS, 0.2 M DTT, 50 mM Tris-Cl, pH 8.0) supplemented with 1% (w/v) plant protease inhibitor cocktail (Bio-Rad, Carlsbad, CA). After incubation at room temperature for 30

min, the suspension was transferred to 1.5 mL eppendorf tube and centrifuged at 15,000 rpm for 15 min, at 4°C. The precipitate was resuspended in 1 mM sample buffer II (50 mM $MgCl_2$, 2,000 units/ml DNase, 750 units/ml RNase, 0.5 M Tris-Cl, pH 8.0). After incubation on ice for 30 min, the mixture was centrifuged at 15,000 rpm for 15 min, at 4°C. Then, trichloroacetic acid (TCA) was added to the supernatant to a final concentration of 10% (v/v). Protein was precipitated at -20°C for 50 min. The protein sample was centrifuged at 15,000 rpm at 4°C for 15 min. The supernatant was gently decanted and the remaining pellet was washed five times with cold acetone. The final pellet was allowed to dry in air or *in vaccuo* at 4°C. The resulting powders of leaf were solubilized in Rehydration Buffer containing 9 M urea, 2 M thiourea, 4% (w/v) CHAPS, 60 mM DTT, and IPG buffer (pH 3-10, Amersham Bioscience). The wheat proteins solubilized in Rehydration Buffer were quantified by a modified Bradford method (Ramagli, 1999).

Two-dimensional gel electrophoresis and gel image analysis

The IPG strip (pH 3-10 NL, 18 cm) (GE Healthcare, Boston, MA) was rehydrated at 20°C for 12 hr with 200 µg of protein in 350 µl of Rehydration Buffer. One-dimensional electrofocusing was carried out at 20°C at 200 V for 1 hr, 500 V for 1 hr, 1,000 V for 1 hr and 10,000 V for 13 hr with 50 µA per strip by an Ettan IPGphor IEF System (GE Healthcare, Boston, MA). The strip was equilibrated with Equilibration Buffer consisting of 50 mM Tris-Cl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and a trace amount of BPB. Just prior to electrofocusing, fresh chemicals, 0.1% (w/v) DTT and 0.25% (w/v) idoaceteamide (IAA) were added to Equilibration Buffer and were allowed to incubate at room temperature for 15 min. The isoelectrofocused proteins on strip gel were separated on 13% (w/v) SDS-PAGE gel on Ettan DALT System (GE Healthcare, Boston, MA) until the BPB reached the bottom of the 2D-gel plate. After the electrophoresis, the 2D-gels were stained with Silver Staining Kit (GE Healthcare, Boston, MA). Silver-stained gels were scanned using flatbed scanner (Powerlook III, UMAX, Seoul, Korea) and 2D-gel images were analyzed using Progress Workstation version 2005 (Nonlinear Dynamics, Newcastle-upon-Tyne, UK).

In-gel digestion

The stained protein spots on 2D-gels, 216 spots in total, were excised with yellow tips and transferred to new eppendorf tube. Then, the gel slice contained protein was subjected to in-gel trypsin digestion (Promega, Madison, WI, USA) as described previously (Shevchenko et al., 1996; Kamal et al., 2011). The gels were destained with a solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate, where the color would change from brown to yellow. The gels were then rinsed a few times with distilled water to stop the destaining reaction and squeezed by acetonitrile. After drying using Speed Vacuum Concentrator (Savant, Holbrook, NY), the gels were incubated with 10 mM DTT and 100 mM ammonium bicarbonate (ABC) at 56°C to reduce the proteins followed by 50 mM iodoacetamide and 100 mM ABC to alkylate the cysteines. The gels were vortexed and completely dried in Speed Vacuum Concentrator. The gel pieces were minced, lypolized, and then rehydrated in 50 mM NH4CO3 with 11.9 ng/µl sequencing-grade modified trypsin (Promega Corp.) at 37°C overnight for 16 h. The peptides were recovered by extraction step using 50 mM ABC and 50% (v/v) acetonitrile containing 0.1% (v/v) formic acid. The resulting peptide extracts were pooled and dried in Speed Vacuum Concentrator. The dried samples were stored at -20°C prior to mass spectrometry analysis.

Matrix assisted laser desorption/ionization (MALDI) analysis

Tryptic peptides were dissolved in 0.5% (v/v) trifluoroacetic acid (TFA), and then desalted by ZipTip C_{18} (Millipore, Bedford, MA). Purified peptides were eluted directly onto a MALDI plate using α-cyano-4-hydroxy-cinnamic acid (CHCA) matrix solution (10 mg/mL CHCA in 0.5% (v/v) TFA/50% (v/v) acetonitrile, 1:1). All mass spectra were acquired in the reflection mode using a 4700 Proteomics Analyzer (Applied Bio-systems, Framingham, MA). External calibration was performed using a standard peptide mixture of des-Arg bradykinin, angiotensin, Glu-fibrinopeptide B, adrenocorticotropic hormone (ACTH) clip 1-17, ACTH clip 18-39, and ACTH clip 7-38. Since wheat genomic database is incomplete, the peptide mass fingerprinting search based on MS data from MALDI-TOF MS not provided sufficient information for identifying protein (Choi et al., 2008, Kamal et al., 2010b). Further mass spectrometry analysis such as MS/MS fragmentation of selected tryptic peptide molecular ions ($[M+H]^+$) was applied to identify the protein by homology searches against non-redundant BLAST database.

Bioinformatic analysis

The protein identification by MS or MS/MS spectra was first conducted by searching homemade local NCBI's wheat (*Triticum aestivum,* 35,536 entries) and rye (*Secale cereale,* 659 entries) non-redundant database using Mascot program (http://www.matrixscience.com, Matrixscience, London, UK). If the protein was not identified in wheat/rye NCBI database, the database search was extended to search the database against rice (*Oryza sativa,* 302,230 entries) and other plant taxonomical entries such as *Arabidopsis* and so on. The search parameters were allowed for N-terminal Gln to pyroGlu, acetylation of protein N-terminus, carbamidomethylation of cysteine as fixed modification and oxidation of Met as variable modification. Mass tolerance of the precursor ion was ± 25- 50 ppm; that of the fragment ions, 0.5 Da; threshold level, 0.05; missed cleavages, 1. The instrument setting was specified as 'MALDI-TOF and MALDI-TOF/TOF'. Protein hits were validated if the identification involved at least 10 top-ranking peptides with *p*-values <0.05 and probability (E-value) less than 0.1, and also selected false positive rate < 0.05. The peptide mass tolerance was less than 25 ppm between theoretical and experimental peptide mass. When those peptides matched multiple members of a protein family, the presented protein was selected based on the highest score and the greatest number of matching peptides. The positive identification had to meet the following criteria: a significant MASCOT score and at least two matched peptides in MS/MS analysis. For functional classification based on gene ontology, we utilized the Protein Information Resources, or PIR (http://pir.georgetown.edu) coupled to NCBI (BLASTtx) database. This integrated public bioinformatics source supports genomics, proteomics, and systems biology research. It is used for determining gene ontology-based molecular functions, cellular components, and biological processes, which are then automatically classified in the data set according to biological process per Batch Retrieval with the *i*ProClass database (Huang et al., 2003).

Conclusion

The proteomics approach was demonstrated to elucidate the protein expression profiles of wheat leaf and to identify the specifically differential expressed proteins by the 2RL-derived translocation lines. Out of 24 differential expressed proteins, 4 proteins were up-regulated, 9 proteins were down-regulated and exclusively expressed in both, and also 2 proteins were disappeared in NIL. Interestingly, cell division protease ftsH homolog proteins were up and down-regulated while Rubisco large subunit were found as down-regulated, exclusive, and disappeared in NIL. As a final point, β-glucosidase, originated from the chromosome 2RL of rye, was exclusively appeared in NIL and small Ras-related GTP binding-protein hit by wheat database as a possible marker protein, whereas acyl-[acyl-carrier-protein] desaturase, chloroplast precursor disappeared in NIL.

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