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Comparative proteomic analyses provide novel insights into the effects of grafting wound and hetero-grafting *per se* on bottle gourd

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ABSTRACT

Grafting is a technology widely used in agriculture and biological research. During a hetero-grafting process, cutting wound and hetero-grafting *per se* are the two main stimuli invoking cellular and whole-plant responses. Despite the importance of dissecting the individual effects of the two in adequately interpreting experimental data, no currently published data are dedicated to comparing the effects of hetero-grafting *per se* and grafting wound. In this study, isobaric tags for relative and absolute quantitation (iTRAQ) technique-based proteomic analyses were conducted between various scion-rootstock combinations of bottle gourd. Our results demonstrated that effects of grafting wound and hetero-grafting *per se* were readily distinguishable at the proteome level. Grafting wound affected the proteomes differentially at the graft union and its upper vicinity. Its effects were also likely related to the stress resistance level of each genotype. The effects of hetero-grafting *per se* on proteome were markedly different between graft union and the upper vicinity. This study provides an explicit evidence for the effectiveness of the commonly used methods of eliminating the effects of grafting wound in a hetero-grafting system by setting up a homo-grafted control. The role of hydrogen peroxide as a signal molecule in hetero-grafting systems is suggested.

1. Introduction

Grafting is a commonly applied technology in vegetable and woody fruit plant production, which has the advantage of conferring the upper part of the graft (known as the scion) improved vigor, productivity and enhanced resistance to various biotic and abiotic stresses (Flores et al., 2010; Schwarz et al., 2010; Huang et al., 2010). Grafting is also widely used as a means to investigate cell-to-cell communication and long-distance signaling between the rootstock and the scion (Kassaw and Frugoli, 2012; Corbesier et al., 2007; Notaguchi et al., 2008; Pina and Errea, 2005; Pina et al., 2012). Technically, grafting is a two-step process comprising incision of the scion/rootstock and reunion of the two at the graft junction. A successful graft is marked by the complete fusion of the graft junction and reestablishment of vascular continuity (Fuentes et al., 2014; Goldschmidt, 2014). Depending on the compatibility between the rootstock and the scion, grafting can be made intra-specific (rootstock and scion belonging to the same botanical species), inter-specific (rootstock and scion belonging to different species of the same genus) or even inter-generic (Mudge et al., 2009).

Rootstock is known to play a critical role in conferring graft advantage. Cookson and Ollat (2013) reported that hetero-grafting, but not homo-grafting, had a major effect on expression of shoot apex genes. Rootstock-derived regulatory RNAs transmission to the scion is considered an important mechanism conferring disease resistance (Ali et al., 2013). Following hetero-grafting with a rootstock, various aspects of plant behavior such as water absorption, nutrient uptake, hormone metabolism and protective enzyme activity may be altered (Liu et al., 2014). However, ascribing such physiological or molecular changes to the effect of hetero-grafting *per se* needs caution, as grafting is always accompanied with cutting wound, an abiotic stress known to trigger multiple cellular responses easily confounding the effects of grafting *per se* (Irisarri et al., 2015; Clemente Moreno et al., 2014; Fluhr., 2001; Turnbull et al., 2002; Schilmiller and Howe, 2005; Stegemann and Bock, 2009). A thorough solution to the confounding effects of wounding was to monitor the cellular events occurring at early stages of graft union development using novel in vitro systems such as micro-callus suspension cultures (Prinsi et al., 2015; Moore and Walker, 1983). Alternatively, some researchers take the method of sampling the plant tissues long after grafting in order to 'dilute' the effect of cutting wound (Liu et al., 2014), while others prefer to set

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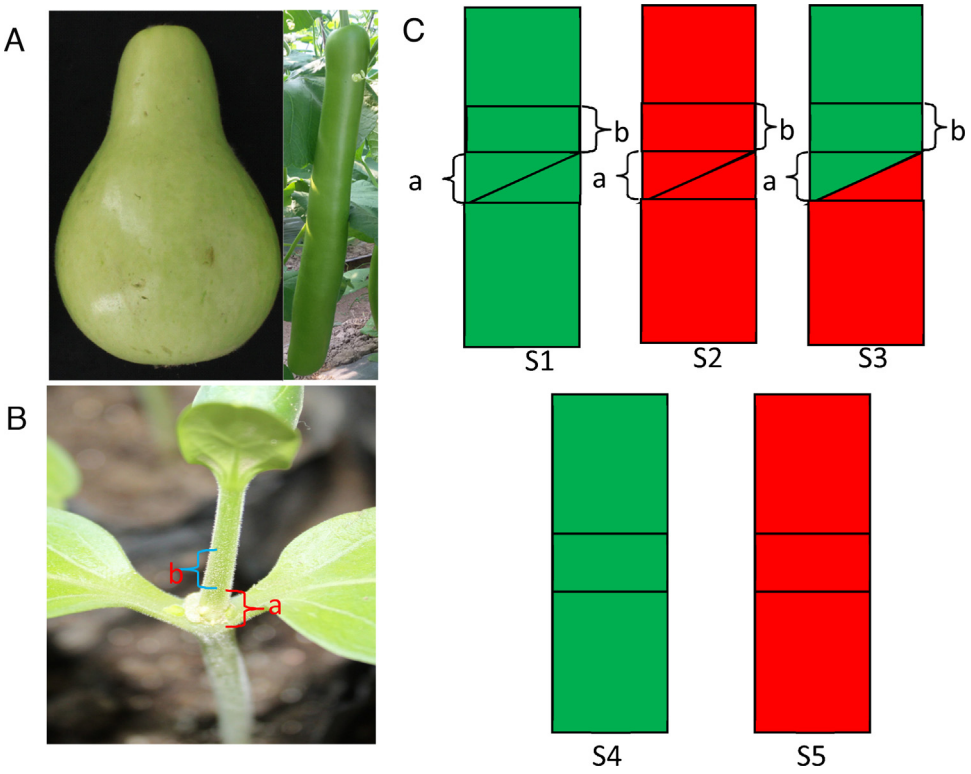

Fig. 1. Plant materials and experimental design for this study. (A) Fruit phenotypes of YZ (left) and HC (right). (B) Graft union (a) and the upper vicinity (b) that were sampled for proteomic analyses. (C) Illustration of the different scion-rootstock combinations. S1: HC/HC homo-grafts; S2: YZ/YZ homo-grafts; S3: HC/YZ hetero-grafts; S4: ungrafted HC; S5: ungrafted YZ.

up a homo-grafted control (Cookson and Ollat, 2013). However, to our knowledge, there are no currently published data using proteomic approaches justifying the effective of the later methods in discriminating the effects of hetero-graft *per se* from cutting wound in vegetable crops.

Proteins are effector molecules in cells that are more directly related to biological processes than mRNAs (Zieske., 2006). Whereas great progress has been achieved in graft biology, relatively few studies are from the proteomic perspective. Isobaric tags for relative and absolute quantitation (iTRAQ), a recently developed proteomic technique, over-performs traditional 2D-gel technique in many aspects including sensitivity, precision and sample throughput (Komatsu et al., 2014; Wu et al., 2006; Wiese et al., 2007). In this study, iTRAQ coupled with MS mass was employed to compare the proteomic profiles of hetero-grafted, homo-grafted and non-grafted bottle gourd plants. Our results demonstrated that effects of grafting wound and hetero-grafting *per se* were readily distinguishable at the proteome level. We also revealed apparent tissue and genotypic differences in the effects of grafting wound and hetero-grafting *per se*. The possible role of hydrogen peroxide as a signal molecule in hetero-grafting systems was highlighted.

2. Materials and methods

2.1. Plant materials

Plant materials used in this study included two bottle gourd varieties i.e. the “Hangzhou gourd” (HC) and “Yongzhen” (YZ). HC is a commercial cultivar with high yield, slender strait fruit shape favored by customers, but susceptibility to major field diseases including fusarium wilt and powdery mildew. YZ, on the contrary, is a landrace cultivar with pear-shaped fruits, high resistance to fusarium wilt and low temperature (Fig. 1A). Due to its excellent stress resistances, YZ is widely used as a rootstock for grafting with watermelon, cucumber and other bottle gourd genotypes in industry (Liu et al., 2013; Zhang et al., 2012). Both HC and YZ are inbred lines and their grafting survival rate is almost 100%.

2.2. Growth conditions and grafting manipulations

Seeds of each variety were sown in plastic pots (6 cm in diameter) filled with sterilized peat soil. After seed germination, the seedlings were kept in greenhouse under an ambient daily temperature of 30 °C and nightly temperature of 27 °C. Natural light/dark circles and normal water management were applied. The grafting was made at the two true-leaf stage using a slit-grafting method (Cushman., 2006). After inserting the trimmed scions into the slits of the rootstocks, the pots were enwrapped with transparent polyethylene bags and maintained for 7 days until sampling, a duration that is known to be enough for the graft union regaining cell adhesion between the two graft partners (Zhang et al., 2011; Hartmann et al., 2002). The two sampling sites for tissue collection were: the graft union (1 cm in length) and the immediate upper area (1 cm in length) along the stem of scion (Fig. 1B). The rootstock-scion combinations included HC/HC (homo-grafted, S1), YZ/YZ (homo-grafted, S2) and HC/YZ (hetero-grafted, HC was the scion cultivar and YZ was the rootstock cultivar, S3). Tissues were collected from 12 individuals of each combination. Ungrafted HC (S4) and YZ (S5) were also sampled (Fig. 1C). The experiments were repeated three times (on March, May and July of year 2014, respectively), and the tissues collected at each experiment were combined for iTRAQ assay.

2.3. Protein extraction, digestion and enrichment of lysine acetylated peptides

Samples were grinded in liquid nitrogen, transferred to 5-mL centrifuge tube and sonicated three times on ice using a high inten-

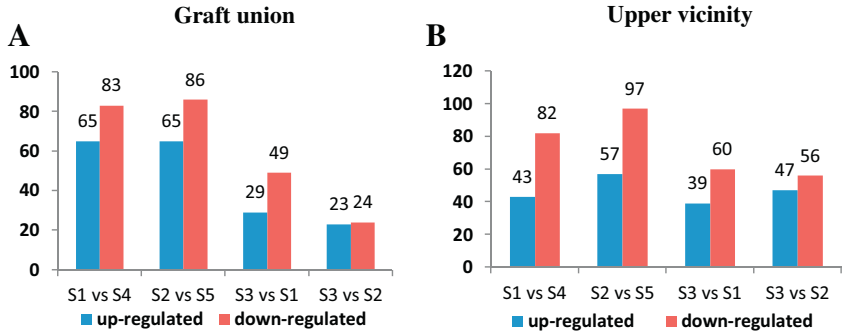

Fig. 2. Numbers of differentially expressed proteins in different rootstock-scion combinations on the 7th day after grafting. (A) Results from the graft union. (B) Results from the upper vicinity.

sity ultrasonic processor (Scientz) in the lysis buffer (8 M urea, 1% Triton-100, 10 mM DTT, 50 mM Tris–HCl and 1% Protease Inhibitor Cocktail). After removing cell debris by centrifugation at 20,000 g at 4 °C for 10 min, the proteins were precipitated with cold 15% TCA for 2 h at −20 °C. The supernatant was discarded after a further centrifugation at 4 °C for 10 min. The precipitate was washed with cold acetone for three times. The proteins were redissolved in the buffer (8 M urea, 100 mM TEAB, pH 8.0) and the protein concentration was determined using a 2-D Quant kit according to the manufacturer's instructions (GE Healthcare, USA).

For digestion, the protein solution was first reduced with 10 mM DTT for 1 h at 37 °C and alkylated with 20 m Miodoacetamide for 45 min at room temperature in darkness. For trypsin digestion, the protein samples were diluted by adding 100 mM buffer to a concentration of less than 2 M. Trypsin was added at an 1:50 trypsin-to-protein mass ratio for the first digestion (overnight) and 1:100 ratio for the second digestion (4 h). Approximately 100 μg proteins for each sample were digested.

2.4. Q-exactive LC-ESI-MS/MS analysis

After trypsin digestion, the peptides were desalted by Strata X C18 SPE column (Phenomenex) and vacuum-dried, then were reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol with 8-plex iTRAQ kit (AB SCIEX).

The samples were then fractionated by high pH reverse-phase HPLC using the Agilent 300 Extend C18 column (250 mm × 4.6 mm ID, particle size 5 μm). The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using NCE setting as 30; ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 2E4 in the MS survey scan with 30.0 s dynamic exclusion. The electrospray voltage applied was 2.0 kV. Automatic gain control (AGC) was used to prevent overfilling of the ion trap; 5E4 ions were accumulated for generation of MS/MS spectra. For MS scans, the *m/z* scan range was 350 to 1800. Fixed first mass was set as 100 *m/z*.

2.5. Database search and gene ontology (GO) term enrichment analysis

The resulting MS/MS data were processed using Mascot search engine (v.2.3.0). Tandem mass spectra were searched against the in-house *Lagenariasicera* genomics database (available upon request). Trypsin/P was specified as the cleavage enzyme and at most 2 missing cleavages were allowed. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethyl on Cys, iTRAQ-8plex (N-term) and iTRAQ-8plex (K) were specified as the fixed modification and oxidation on Met was specified as the variable modification. For protein identification, the threshold for FDR was set at 0.01 and peptide ion score at 20. The cutoff values for declaring a differentially expressed protein were: fold change >1.3 or <0.77, and the *P*-value ≤ 0.05.

Gene ontology (GO) enrichment analyses were performed using Gorilla (Eden et al., 2009), under a *P*-value threshold of $10E^{-4}$ for statistical significance. Prior to running the program, the DEPs were subjected to BLASTX searches against *Arabidopsis* protein sequences under a *P*-value cut-off of $10E^{-3}$ to provide legible sequence IDs for recognition in the program.

3. Results

3.1. Grafting wound affected the proteomes differentially at the graft union and its upper vicinity

As a homo-grafted plant and a non-grafted plant differ only in whether or not they were subjected to cutting (followed by reunion), we at first performed a comparative proteomic analysis between the non-grafted and homo-grafted plants in order to elucidate the effects of grafting wound. At the graft union, a very close number (148 VS 151) of differentially expressed proteins (hereafter, DEPs) were identified from the two genotypes (Fig. 2A, Table S1). Among them, more than 50% of the induced and 70% of the suppressed proteins were shared between the two genotypes. Gene ontology (GO) enrichment analysis unraveled that, as expected, GO terms related to wound responses and defense signaling, such as "response to stimulus", "phenol-containing compound metabolic process", "oxidoreduction coenzyme metabolic process", and "salicylic acid metabolic process" were the predominantly enriched categories in both genotypes (Table S2). Besides these common GO terms, several GO terms related to nucleic acid metabolism (e.g." nucleotide phosphorylation", "ribonucleotide metabolic process") and immune (e.g. "regulation of immune response" and "regulation of immune system process") were enriched in a genotype-specific manner.

At the upper vicinity of graft union, nearly twice the number of induced than suppressed proteins were found in both genotypes (Fig. 2B, Table S3). The GO term "Response to abiotic stimulus 'still was significantly enriched in both genotypes, reflecting the impact of wounding at this area even though it was only indirectly related to grafting wound. More GO terms enriched at this area were indeed not tightly associated with wound and their distribution showed interesting genotypic specificity, for example "response to blue light', 'response to red light' and "jasmonic acid mediated signaling pathway" were only enriched in HC, while "regulation of

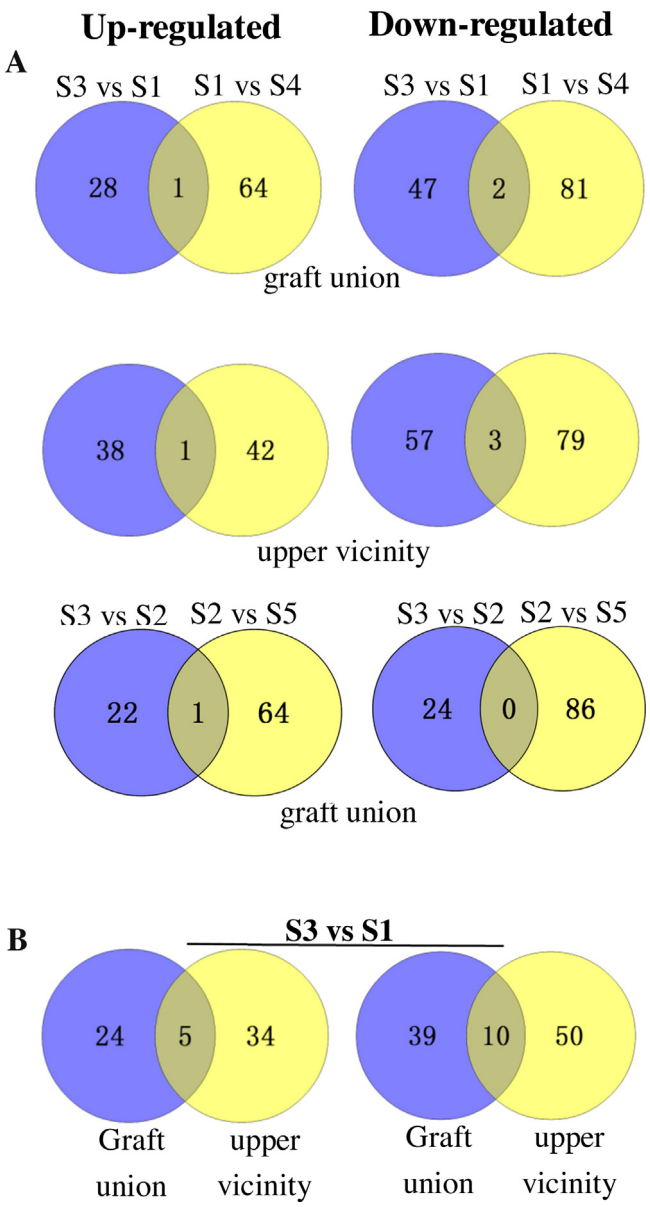

Fig. 3. Venn diagram of the number and relationship of differentially expressed proteins between hetero- and homo-grafted plants. (A) Comparison between S1 and S3 at different sites and between S2 and S3 at the graft union. (B) Comparison between graft union and the upper vicinity in S3.

programmed cell death" and "regulation of plant-type hypersensitive response" were only enriched in YZ (Table S4).

3.2. *Effects of grafting wound and hetero-grafting* per se *were readily distinguishable at the proteome level*

We then compared the DEPs between S1 and S4 (effect of grafting wound only) to those between hetero-grafts (S3) and homo-grafts (S1, S2). As the effects in the graft combination S3 consisted of both grafting wound and genotypic interaction (grafting *per se*), such comparison should be able to reveal the differences of the two effectors. As shown in Fig. 2, much less DEPs were found between S3/S1 than between S1/S4, particularly at the graft union. The same trend was noticed between S3/S2 than between S2/S5 at the graft union. More importantly, in both tissues the DEPs due to grafting wound (S1/S4, S2/S5) were almost totally different from those between heterografts (S3/S1, S3/S2) (Fig. 3A), strongly indicating that the effects of grafting wound and hetero-grafting *per se* were readily distinguishable at the proteome level. These results also provide an explicit evidence for the effectiveness of the methods of using homo-grafted control to eliminate the effects of grafting wound in a hetero-grafting system.

3.3. *Effects of hetero-grafting* per se *on proteome were markedly different between graft union and the upper region*

We then sought to characterize the effects of hetero-grafting *per se* in more details by deeply analyzing the DEPs between S3 and S1. In general, we found the effect of hetero-grafting *per se* more significant in the upper vicinity than in graft union as there were considerably more DEPs in the former (Table S5). Moreover, only a small portion of the DEPs were in common between the two tissues (Fig. 3B), such as "heat shock 70 kDa protein", "UDP-glucose 6-dehydrogenase", "F-box/LRR-repeat protein" and "Beta-D-xylosidase 4". Four GO terms, two hydrogen peroxide-related, were enriched at the graft union. At the upper vicinity, on the contrary, there was no GO term significantly enriched (Fig. 4).

4. Discussion

Graft establishment is a complicated process involving wound/healing processes, division/differentiation, cell-to-cell communication and vascular regeneration (Yin et al., 2012; Cookson et al., 2014). Our results revealed major differences in grafting wound-induced DEP patterns between graft union and the upper vicinity. Wound stress related GO terms were the predominantly enriched at the graft union, consistent with the fact that this area is where the injury/repair occurred. In contrast, the change of proteome at the upper vicinity appeared to be an indirect result of wounding triggered by complicated cellular signals. The differential expression of proteins involved in blue and red light signaling pathways provides an evidence for this assumption in that both pathways are known to be cross-linked with abiotic stress responses (Xu et al., 2009). In addition, the marked genotypic differences in DEPs at this area is likely in relation to the differential stress resistance capacities/mechanisms of each genotype. The enrichment of the GO terms "regulation of programmed cell death" and "regulation of plant-type hypersensitive response" specific to YZ is likely a result of its strong resistance to multiple diseases and environmental cues of which the signaling pathways are partly overlapped with graft wounding.

By comparing transcriptomic data between hetero- and homo-grafted grape plants, Cookson and Ollat, 2013; Cookson et al., 2014 concluded that expressions of many defense-related genes in the shoot apex were affected by grafting *per se*, based on the observation of coordinated upregulation of numerous stress responsive genes in the hetero- compared to the autografts. However, this seems not the case in our study, at least at the proteomic level. Our datas show that defense responses are more likely a result of grafting wound. Such difference may be partly attributed to the high compatibility between the rootstock/scion combination used in our study, different sampling sites, durations from grafting to tissue collection, or the inconsistency between the transcriptome and the proteome. Nevertheless, we still detected DEPs, albeit few, that are likely associated with defense responses, such as an F-box/LRR-repeat protein and the Glutathione S-transferase L2. We speculate that in a hetero-grafting system where genetic heterogeneity between the scion and the rootstock is present, defense-like responses would also be triggered at the graft union. This assumption is further supported by the finding of the enrichment of GO terms related to hydrogen peroxide, a well known component of defense signaling pathways (Hung et al., 2005). Previously, alter-

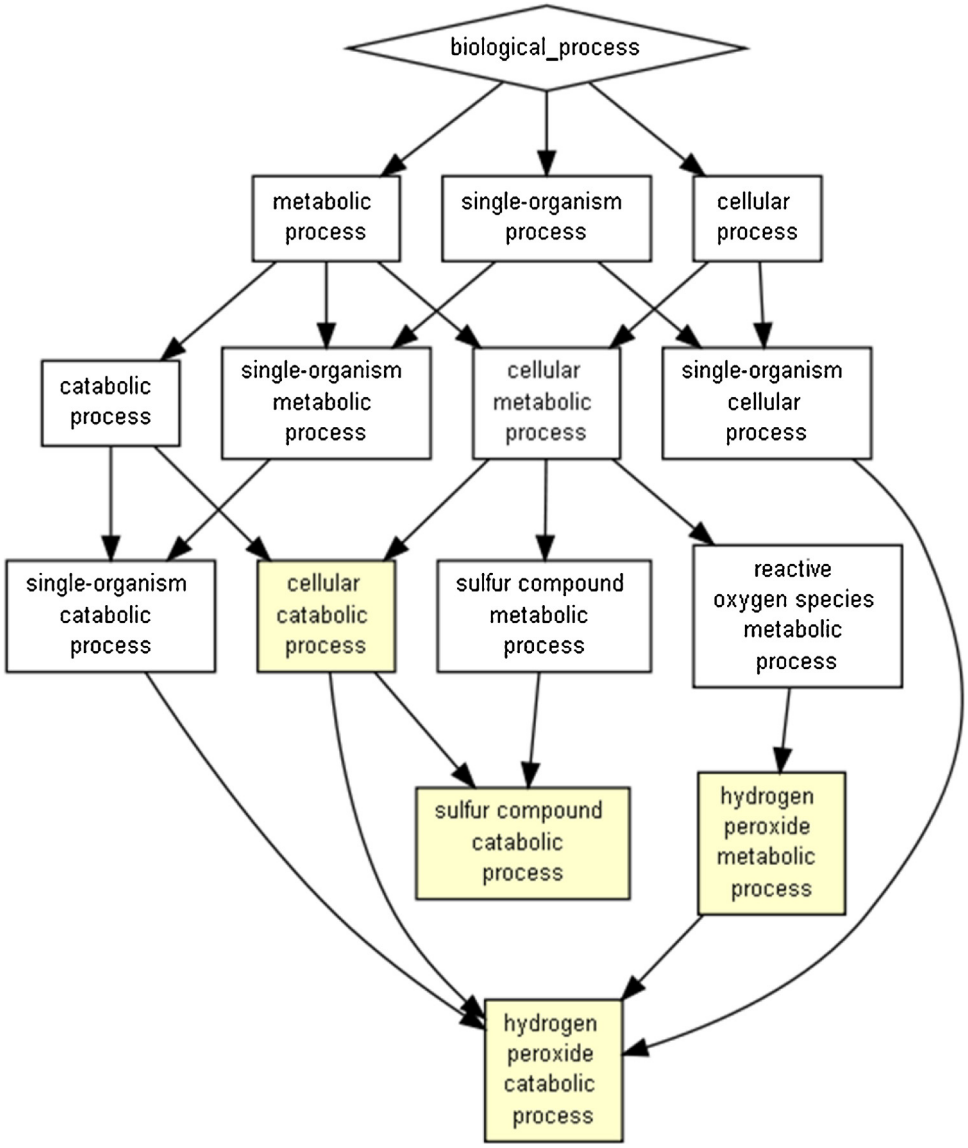

Fig. 4. Schematic show of the enriched GO terms between S3 and S1 at the graft union.

ation of peroxidase and catalase activities in hetero-graft unions were reported (Fernández-García et al., 2004). The role of hydrogen peroxide as a signal molecule in incompatible interaction and hypersensitive reactions has also been well established (Hung et al., 2005). More recently, hydrogen peroxide mediated HSP70 accumulation and heat tolerance in grafted cucumber plants via ABA signaling was also uncovered (Li et al., 2014). It is an interesting future task to more thoroughly characterize the role of hydrogen peroxide in scion-rootstock interaction in hetero-graft systems.

Our results also provide interesting clues in relation to the early stages of graft development, especially in the hetero-grafts. For example, photosynthesis related proteins are known to be important in plant growth and development (Hu et al., 2015). In our study, the photosystem II reaction center protein (psbB), photosystem II D2 protein (psbD) and the electron carrier protein (psbE) were up-regulated at the upper vicinity in hetero-grafts only; some other proteins such as photosystem II reaction center protein C (psbC), the photosynthetic electron transporter (petB) were down-regulated in auto-grafts. Some enzymes involved in carbohydrate and amino acid metabolism were also differentially regulated between hetero-grafts and auto-grafts. We observed that cysteine synthase and phenylalanine ammonia-lyase (PAL) and aspartate aminotransferase were up-regulated at the graft interface in hetero-grafts only. PAL has been linked to biosynthesis of many secondary metabolites such as anthocyanins, flavanols, and lignins (Duke et al., 2006). In addition, carbohydrate metabolism-related proteins such as fructose-bisphosphate aldolase and malate dehydrogenase (Hirosato et al., 2004; Beeler et al., 2014) were specifically up-regulated in hetero-grafts only at the graft union. These results taken together indicate a complicated and coordinated regulation network at the proteome level that fosters the establishment of successful hetero-grafts and gain of hetero-grafting advantages.

5. Conclusion

The effects of grafting wound and hetero-grafting *per se* are distinguishable and quite different at the proteome level, justifying the effectiveness of the commonly used methods of using homo-grafted control to eliminate the effects of grafting wound in a hetero-grafting system. Both grafting wound and hetero-grafting *per se* affect the proteomes differentially at the graft union and its upper vicinity. Grafting wound as an abiotic stress evokes resistance/defense signals related to the field disease/abiotic resistance

level of the subjected genotypes. Hydrogen peroxide might serve as a signal molecule in hetero-grafting systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scienta.2015.12.056.

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