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BRIEF COMMUNICATION

Protein profile in the transgenic kiwifruit overexpressing a transcription factor gene, *OSH1*

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Abstract

The aim of this study was to investigate the protein alterations in the transgenic kiwifruit (*Actinidia deliciosa*) overexpressing a transcription factor gene, *OSH1*. Although transgenic plant with introduced *OSH1* indicates suppression of gibberellin (GA)-20 oxidase activity, application of GA to transgenic kiwifruit could not completely recover plant morphology and protein profiles. Eleven proteins decreased in the transgenic kiwifruit detected by two-dimensional polyacrylamide gel electrophoresis showed homologies to kiwifruit hypothetical protein, osmotin I and photosynthesis related protein. These results suggest that introduction of an *OSH1* into kiwifruit caused wide-range alterations at protein level and alterations of protein accumulation should be considered to evaluate the substantial equivalence of plants transformed by a transcription factor.

Additional key words: *Actinidia deliciosa*, 2D-PAGE, gibberellin oxidase, gibberellins, proteomics.

In plant kingdom, homeobox genes have been identified in maize, *Arabidopsis*, rice and many other species. They have conserved domains called homeodomain (Gehring 1987) and make similar products containing helix-turn-helix motif (Scott *et al*. 1989). Their products recognize and bind specific DNA sequences. Therefore, homeobox genes are considered as transcription factors to regulate the expression of genes. *OSH1*, one of the homeobox genes, was isolated from rice as a homologous gene to maize homeobox gene *knotted-1* (Matsuoka *et al*. 1993). Transgenic plants overexpressing *OSH1* resulted in shrunk leaves and loss of apical dominance (Kano-Murakami *et al*. 1993). Similar morphological characteristics were also shown in the transgenic kiwifruit which we have already introduced *OSH1* (Kusaba *et al*. 1999). Such morphology in *OSH1*-transformed plants is ascribed to the reduction of GA_1 and GA_4 contents (Kusaba *et al*. 1998). However, their morphology was not completely recovered even though GA_3 and GA_4 were exogenously applied to transgenic kiwifruits (Fig. 1). The length of internode and the width of leaves became relatively longer and wider compared with the transgenic kiwifruit without GA application, respectively. This result suggests that the introduced factor, *OSH1*, affected not only GA metabolism but also unexpected other gene regulation.

Since the plant morphologies, such as dwarfing and branching, are very important characteristics in agriculture for the purpose of saving labour and increasing the planting density in the limited area, production of transgenic plants with transcription factor has been considered as one of the approaches to modify the plant morphology. However, there are few reports how the genes/proteins are affected by a transcription factor so far. Identification of these genes/proteins and predicting the potential occurrence of unanticipated

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Abbreviations: CBB - Coomassie brilliant blue; 2D-PAGE - two-dimensional polyacrylamide gel electrophoresis; GA - gibberellin; IEF - isoelectric focusing; PVDF - polyvinylidene difluoride; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis.
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modification in the transgenic plants with a transcription factor are also required for safety assessment of genetically modified food.

To detect the genes, DNA microarray, gene subtraction and differential screening are available tools. For proteins, high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is powerful by comparisons of the separated protein mixtures between wild and transgenic plants. Because of its high resolvability, this technique has been used to detect the protein alterations accumulated in the process of plant development and in response to various kinds of environmental conditions (Celis and Bravo 1984, Dell'Aquila 2004). Therefore, we compared the protein profiles between *in vitro* cultured *OSH1*-overexpressing transgenic kiwifruit [*Actinidia deliciosa* (A. Chev) C.F. Liang *et* A.R. Ferguson var. *deliciosa* cv. Hayward] and the wild type by 2D-PAGE, and identified the decreased proteins specific to the transgenic kiwifruit.

Proteins were extracted by trichloroacetic acid-acetone treated powder from *in vitro* cultured shoots as described by Damerval *et al*. (1986). Five mg of powder was homogenized with 0.1 cm^3 of lysis buffer containing 8 M urea, 2 % ampholine pH 3.5 - 10, 2 % *Nonidet P-40*, 5 % 2-mercaptoethanol, and 5 % polyvinylpyrrolidone. The supernatant was recovered by centrifugation and subjected to isoelectric focusing (IEF) in the first dimension and sodium dodesyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension, as described by O'Farrell *et al*. (1975). IEF was conducted at 200 V for 30 min, at 400 V for 20 h, and finally at 600 V for 1 h. After IEF, the gel was equilibrated with the SDS-sample buffer and applied to 15 % SDS-PAGE for second dimension electrophoresis. The second dimension electrophoresis was performed at a constant current of 35 mA. Proteins were stained with Coomassie brilliant blue (CBB) R 250. The detection, decision of isoelectric point and molecular mass of each protein and comparison of differences in protein spots between the transgenic kiwifruit and the wild type were determined using software (*ImageMaster 2D Elite*, *Amersham Biosciences*, Piscataway, USA).

Following the separation using 2D-PAGE, the proteins were electro-blotted onto a polyvinylidene difluoride (PVDF) membrane and detected by CBB staining. They were cut out from the membrane and applied to gas-phase protein sequencer (*Precise 494*, *Applied Biosystems*, Foster City, USA). When the N-terminus of protein was blocked, the protein was recovered from the gel pieces by electroelution as described by Konishi *et al*. (2001), and digested by *Staphylococcus aureus* V8 protease during SDS-PAGE as described by Cleveland *et al*. (1977). The fragments were excised from the PVDF membrane and subjected to protein sequencing, as described above.

In the transgenic kiwifruit and the wild type, we obtained about 80 protein spots by CBB staining (Fig. 2*A*,*B*). After normalizing by analysis software, almost the same spot number (about 80 spots) was detected in both transgenic kiwifruit and wild type with high repeatability. In the 80 spots, though no spots were obviously increased in the transgenic kiwifruit, 11 protein spots were found to decrease distinctly (Fig. 2). Therefore, homology of each 11 decreased protein spots

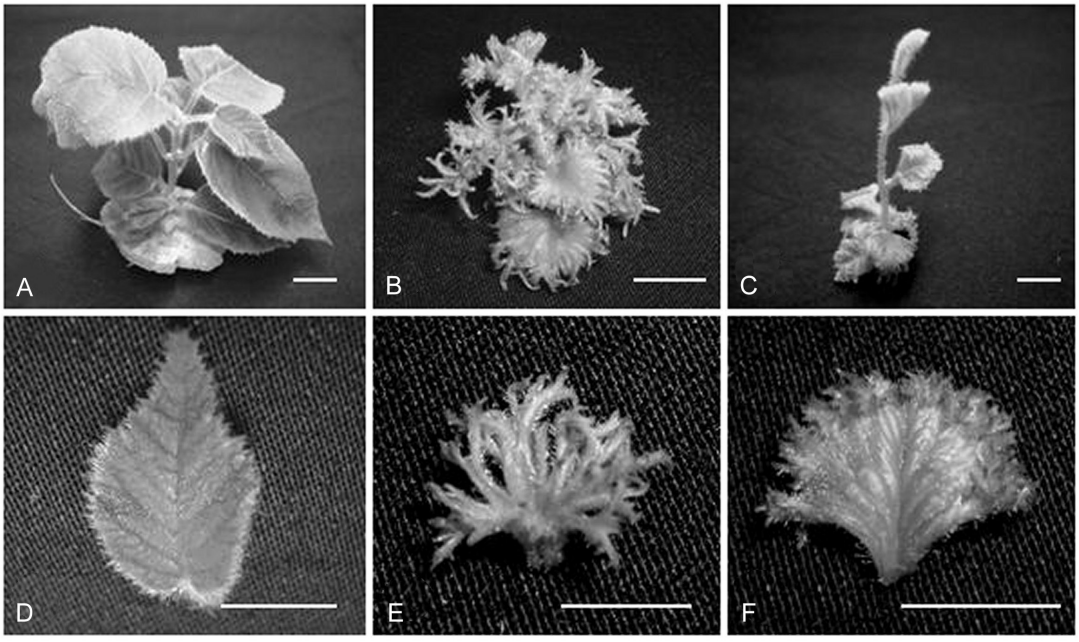

Fig. 1. Shoot (*A*, *B* and *C*) and leaf (*D*, *E*, and F) morphologies of the wild type kiwifruit (*A*, *D*), *OSH1*-overexpressing kiwifruit (*B*, *E*) and *OSH1*-overexpressing kiwifruit after GA_4 treatment (*C*, *F*). *Scale bars* = 1 cm toward the horizontal direction.

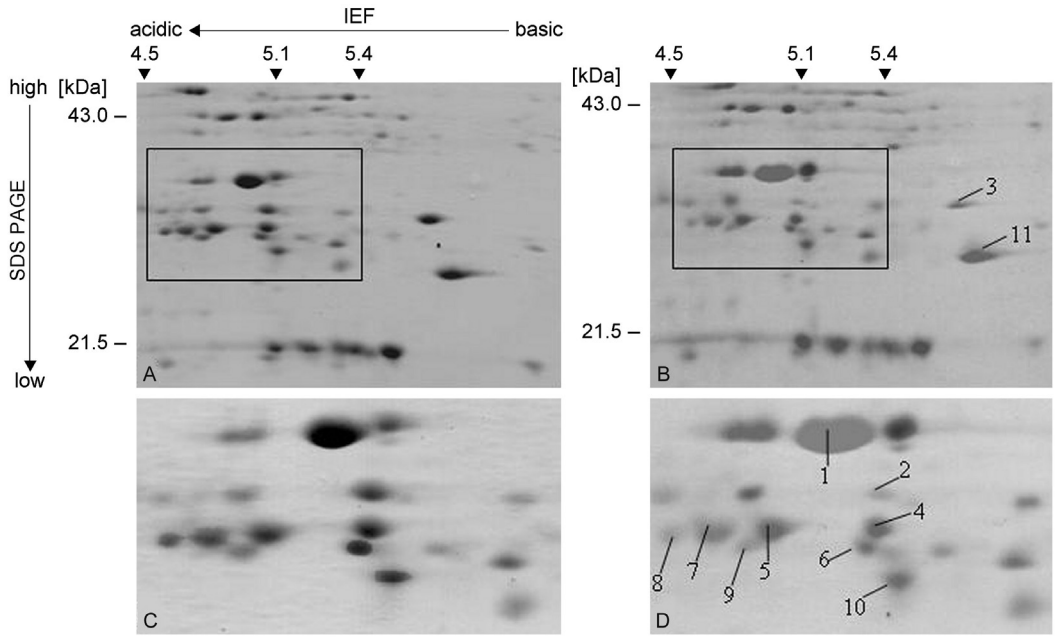

Fig. 2. Protein profiles in the wild type kiwifruit (*A*) and *OSH1*-overexpressing kiwifruit (*B*). The magnification of the analyzed area covered by squares in (*A*) and (*B*) is also present as also (*C*) and (*D*), respectively. The pI value and relative molecular mass of each protein were determined using 2D-PAGE marker (*Bio-Rad*). Proteins that were decreased in the transgenic kiwifruit are numbered in (*B*) and (*D*).

Table 1. Amino acid sequences and other characteristics of the polypeptides whose accumulation were reduced in *OSH1*-overexpressing kiwifruit (* - the amino acid sequences were determined using the Cleveland method).

Spot No.	Amino acid sequence	pI	M_r [kDa]	Homologous protein	Homology [%]
1	EGVPKPLTYD	4.97	33.3	photosystem 2 oxygen evolving complex protein	90.0
2	WAVTQQATTT	5.05	31.4	cytochrome c_5	80.0
3*	AKPTXTEVPL	5.67	30.4	cytochrome f	70.0
4	AIRQDTAIRQ	5.05	29.8	kiwifruit hypothetical protein	90.0
5	AIRQDTAIRQ	4.86	29.1	kiwifruit hypothetical protein	90.0
6*		5.03	29.0	not determined	
7	SILQDTAILQ	4.75	28.8	kiwifruit hypothetical protein	80.0
8*	QFGANKXQY	4.69	28.6	*Homo sapiens* NICE-5 protein	88.9
9*		4.81	28.3	not determined	
10	AANIELRNNXP	5.11	28.0	osmotin-I	72.7
11	AYGEAANKIG	5.73	25.8	photosystem 2 oxygen evolving complex protein	80.0

was searched (Table 1). The amino acid sequences were compared to amino acid sequence database (DDBJ; http://www.ddbj.nig.ac.jp). The amino acid sequences of the spots #6 and #9 could not be determined, although the Cleveland method was employed for the determination of internal sequences. Spots #4, #5 and #7 were homologous to the hypothetical protein pKIWI502, which was expressed strongly in fruit at 8 - 10 d after anthesis (Ledger *et el*. 1994), whose exact role is still unknown. Spot #10 was homologous to a defense-related protein osmotin I, which was induced under osmotic stress condition (Singh *et al*. 1987). Spots #1 and #11 showed homology to photosystem 2 oxygen evolving complex protein. Spots #2 and #3 were cytochrome related proteins. It has been indicated that the proteins related to photosynthesis were quantitatively changed in the GA-treated plants (Komatsu *et al*. 2002), although the exact reason is not yet known. These results suggest that GA and its metabolism may play crucial roles in the proper maintenance of photosynthesis. Spot #8 was homologous to the *Homo sapiens* NICE-5 protein (Marenholz *et al*. 2001). At present, there are no homologous proteins to the NICE-5 protein in the plant database, and to our knowledge, this is the first indication of the NICE-5 homologue in plants.

So far, molecular analysis of transgenic tobacco plant introduced with *OSH1* was reported by Tamaoki *et al.* (1997). They identified 39 up-regulated cDNA clones in

the transgenic tobacco by cDNA subtraction method. However, post-transcriptional and post-translational regulations usually take place after the transcription and translation. Therefore, comparing the protein profiles is considered to reflect the actual changes in the investigation of equivalence between the transgenic plant and the wild type. Moreover, 2D-PAGE analysis can monitor simultaneously both the protein with either increased or decreased.

It has been reported that GA-20 oxidase activity was suppressed in the *OSH1*-overexpressing transgenic plants (Kusaba *et al*. 1998, Sakamoto *et al*. 2001). Although the GA-20 oxidase protein was expected to detect in the predicted position of 2D-PAGE from the wild type and to disappear (or reduce) from the *OSH1*-overexpressing kiwifruit, its corresponding spot was not detected in this study. GA-20 oxidase protein may be present in trace amount or below detectable level, which could not detected by CBB staining method. It has been demonstrated that CBB staining may not be sensitive enough to detect the protein with trace amount (Gygi *et al*. 2000). Though the reason(s) why obviously increased proteins were not detected in this study has not been clear yet, there is a possibility that some protein is increased in the transgenic plant under the detectable level. Therefore, there might be many changes more than the detecting protein spot by CBB staining in the *OSH1*-over-expressing kiwifruit. This might be one of the reasons why increased proteins in the transgenic plant could not be detected in this study. After all, there might be many changes under the detectable level in the *OSH1*-over-expressing kiwifruit.

In conclusion, taking into account of the results from 2D-PAGE and exogenous GA application, it was apparent that the proteins that were affected by *OSH1* were more than we had anticipated. Comparison of the protein by 2D-PAGE is a valuable method to monitor the proteins whose accumulations were modified by the introduction of a transcription factor gene, such as *OSH1*, and the data would be useful for understanding the regulation by a transcription factor and for evaluating the equality (substantial equivalence) in the transgenic plants.

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