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Short Communication

Searching for genetic factors involved in hybrid lethality in *Nicotiana*; Possible use of a cultured cell line of a hybrid

Masanobu Mino[1,*], Mami Kubota[1], Yuki Yamaguchi[1], Tetsu Kawamori[1], Kenji Sato[2], Yuko Misaka[1], Yoshikazu Tanaka[3], Masayoshi Inoue[1]

[1] Graduate School of Agriculture, Kyoto Prefectural University, Sakyo-ku, Kyoto 606-8522, Japan; [2] Department of Food Science and Nutritional Health, Kyoto Prefectural University, Sakyo-ku, Kyoto 606-8522, Japan; [3] The Wakasa-wan Energy Research Center, Tsuruga, Fukui 914-0192, Japan
* E-mail: mino@kpu.ac.jp Tel & Fax: +81-75-703-5604

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Abstract The cell line GTH4 generated from the F_1 hybrid of *Nicotiana gossei*×*N. tabacum* reveals lethality at the seedling stage. The cells proliferated at 37°C but died at 26°C. GTH4S, a cell line derived from GTH4 that does not die at 26°C, was established. Differences in the genes and proteins expressed in the two cell lines were analyzed. GTH4 expressed the gene encoding ubiquitin-conjugating enzyme (E2) which was not expressed in GTH4S. PCR targeting this sequence indicated that GTH4S is missing a segment of DNA containing the *E2* locus, suggesting that GTH4S is a deletion mutant of GTH4. An analysis of proteins using anion-exchange HPLC and SDS-PAGE revealed GTH4 to contain protein species not found in GTH4S. The genes and/or proteins involved in the expression of hybrid lethality can be analyzed by comparing these two cell lines.

Key words: Cultured cells, differential display, hybrid lethality, *Nicotiana*, SDS-PAGE.

Hybrid lethality is a process of reproductive isolation which splits a species into two. The unraveling of this major driving force of evolution is a central topic in the evolutionary genetics of speciation. In animals, genetic factors which might be involved in hybrid lethality have been reported: the genes encoding tyrosine kinase in platy fish (Wittbrodt et al. 1989), a protein with two MADF DNA-binding domains (MYB-related domain) (Barbash et al. 2003), and nuclear pore proteins in *Drosophila* (Presgraves et al. 2003). However, little information is available on hybrid problem genes in plants. The discovery of these genes is important not only for studies on evolutionary genetics in plants but also for plant breeding using wide crosses.

In an attempt to elucidate the mechanism of hybrid lethality, we have studied the physiological and biochemical aspects of the death of hybrids; Cell death in seedlings of the F_1 hybrid of *Nicotiana gossei* Domin and *N. tabacum* L. proceeded with a rapid collapse of cellular components (Mino et al. 2002). The cell death proceeded at 26°C but was suppressed at 37°C, and hydrogen peroxide functioned as a mediator to control the cell death process (Mino et al. 2004). A cell line, GTH4, of this hybrid proliferated normally at 37°C, but started to die after a shift in temperature from 37°C to 26°C. Analyses using GTH4 indicated a loss of vacuolar functions to be a key executioner of the cell death (Mino et al. 2005). We recently established a cell line from GTH4 which does not die at 26°C. This line, GTH4S, would be useful for studying the biochemical, physiological, and molecular aspects of hybrid lethality in comparison with GTH4. Here, we briefly report the results of recent studies on hybrid lethality using these cell lines.

After the prolonged culture of GTH4 at 26°C, calli were occasionally generated around dead cells and could be proliferated for generations. However, these cells died at 26°C after a period at 37°C, suggesting that they were a phenocopy of non-lethal cells. GTH4S, by contrast, did not die at 26°C after a period at 37°C (Figure 1). The experiment where the temperature was lowered from 37°C to 26°C was conducted several times, but no cell death reaction was observed. We therefore concluded that GTH4S was non-lethal at an impermissible temperature. The shape and volume of cells did not differ between the two lines, but GTH4S was a slightly stronger green than GTH4 (Figure 1B).

Treatment of the GTH4 cells with actinomycine D and

Abbreviations: HPLC; High performance liquid chromatography, RT-PCR; Reverse transcription-polymerase chain reaction, SDS-PAGE; SDS-polyacrylamide gel electrophoresis, UTR; untranslated region.
This article can be found at http://www.jspcmb.jp/

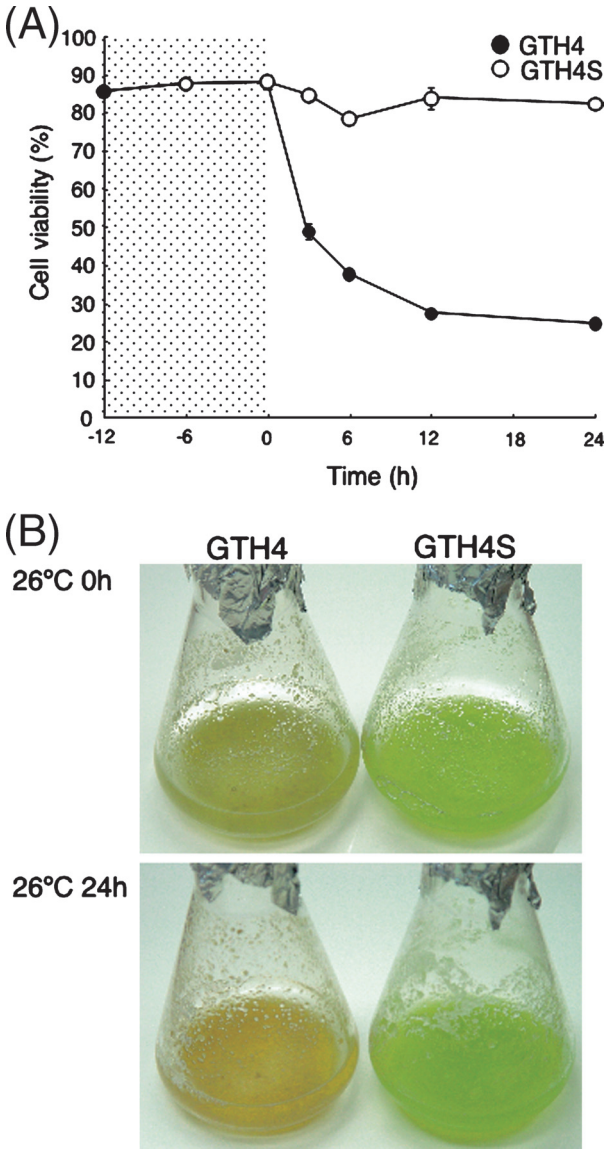

Figure 1. Changes in viability of two cultured cell lines, GTH4 and GTH4S, in the hybrid of *N. gossei*×*N. tabacum* in response to a shift in temperature from 37°C to 26°C. (A) The temperature in the period indicated by hatching is 37°C, otherwise it is 26°C. The viability of cells was determined using FDA as described previously (Mino et al. 2005). The percentage data were transformed to arcsine values for calculation of the standard error (SE) of the mean. At least 200 cells were observed for each replication. Vertical bars are SE (n=5). (B) Features of the cultured cell lines before and after the shift of temperature from 37°C to 26°C.

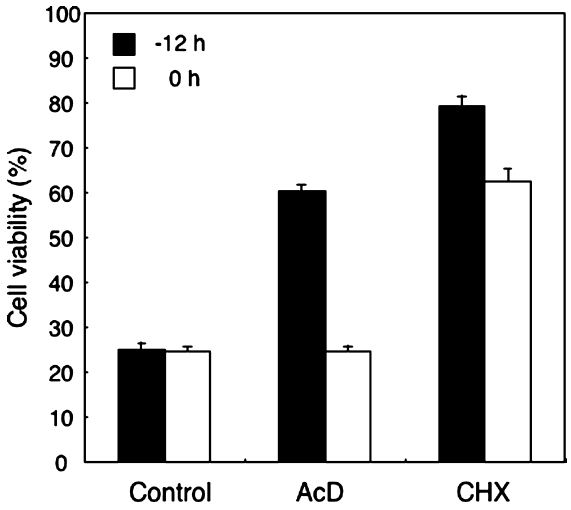

Figure 2. Effects of inhibitors of RNA and protein synthesis on the viability of the cells of GTH4. 25 μM of actinomycin D (AcD) or 10 μM of cycloheximide (CHX) was applied to the cells 12 or 0 h before the shift in temperature from 37°C to 26°C, and cell viability was determined 24 h after the shift. Vertical bars are SE (n=5). At least 200 cells were observed for each replication.

cycloheximide 12 h before the temperature shift suppressed cell death to a greater extent than did treatment at 0 h, suggesting that the RNAs and proteins required for cell death were synthesized during the culture at 37°C (Figure 2). However, cell death is conditionally suppressed by an unknown mechanism at the permissible temperature. The treatment with cycloheximde at 0 h still inhibited cell death, suggesting that the protein synthesized in the cells after the temperature shift is active in advancing the cell death process at 26°C. These results suggested that GTH4S did not express the genes and/or synthesize the proteins, which were involved in the cell death of GTH4.

Since there might be a functional relationship between the gene expression and cell death in GTH4, it was worth comparing the differences in the genes expressed between GTH4 and GTH4S. To detect the differences, RNAs prepared from both cell lines cultured at 37°C were subjected to a differential display analysis (Liang and Pardee 1992). Twenty-four combinations of down-stream anchor primers (3) and up-stream random primers (8) were used for the analysis. The amplicons generated in the PCR were separated on a thin-layer urea-polyacrylamide gel and detected using a silver stain kit (Silver Stain KANTO III, KANTO CHEMICAL Co., Inc., Tokyo Japan). After two rounds of re-amplification, an amplicon which was specific to GTH4 was cloned into the pBS II SK(+) plasmid vector (Stratagene) and sequenced. The full-length cDNA clone of this gene was obtained from a λTriplEx2 cDNA library (Clontech) constructed from poly (A+) RNA of GTH4 cells grown at 37°C. This gene encodes ubiquitin-conjugating enzyme (E2) (Figure 3). RT-PCR revealed that *E2* was expressed in GTH4, but not in GTH4S (Figure 4A). An analysis of genomic DNA suggested that all or part of this locus was missing from the genome of GTH4S (Figure 4B). An analysis of *N. tabacum* (genomic composition: TTSS), its ancestral parent species *N. tometosiformis* (TT) and *N. sylvestris* (SS), and *N. gossei* revealed that this locus was derived from *N. tometosiformis*, not from *N. sylvestris* or *N. gossei*. In the ubiquitin-proteasome pathway, E2 transfers activated ubiquitin to a member of the ubiquitin-protein ligase

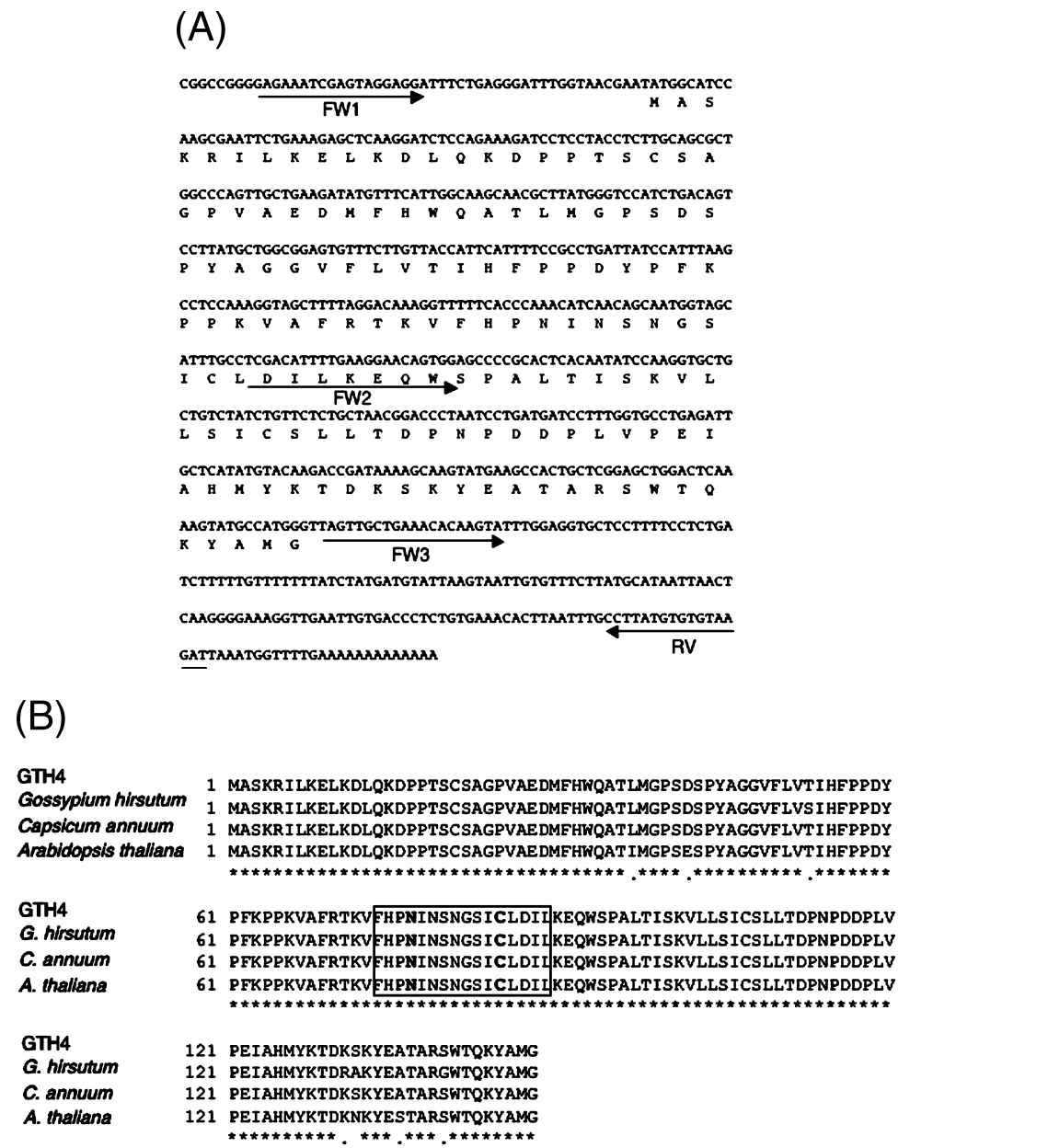

Figure 3. The gene encoding ubiquitin-conjugating enzyme (E2) of GTH4. (A) The sequence of cDNA and deduced open reading frame. Arrows indicate the site of primers used for the PCR (cf. Figure 4). (B) Comparison of the amino acid sequence of E2 of GTH4 and other plants. *G. arboreum* (accession number: AY082005), *C. annuum* (DQ249816), *A. thaliana* (DQ027024). The active site motif is boxed. The active site cysteine, which forms the thiol ester with ubiquitin, is shown in bold. Asparagine in bold upstream of cysteine plays a role in stabilizing the E2 structure (Bernier-Villamor et al. 2002), and is conserved. Asterisks and dots represent complete and much similarity, respectively.

family, E3 (Ciechanover 1998). This protein degradation system plays a role in many cellular activities including the regulation of metabolism and signaling pathways (Agetsuma et al. 2005; Dill et al. 2004; Fu et al. 2004), and its impairment would be deleterious to many cellular functions. Individual members of the conserved family of E2 mediate degradation of specific substrates in the ubiquitin-dependent proteolytic pathway in a cell. The specificity of the E2-E3 complex was also confirmed by an in vitro ubiqutination assay (Kraft et al. 2005). However, it was reported that the genome of *Saccharomyces cerevisiase* encodes for 13 E2s and E2-like proteins (Ciechanover 1998), and 14 E2 proteins were found in Arabidopsis (*Arabidopsis thaliana*) (Liu and Podila 2000). More recently, inspection of E2 in the Arabidopsis genome revealed that there are at least 37 ubiquitin conjugating (UBC) domain-containing proteins, which were further divided into 16 subgroups according to a phylogenetic analysis (Kraft et al. 2005). The amino acid sequence of E2 of GTH4 was 97% identical to that of AtUBC10 (GenBank accession number: DQ027024) of Arabidopsis (Figure 3B).

AtUBC10 belongs to subgroup 6, the largest Arabidopsis E2 subgroup, and the proteins in this subgroup are similar to human HsUBC5a to c and yeast ScUBC4 and 5 (Kraft et al. 2005). All this suggests that multiple loci of this gene are present in the genome of F_1 hybrid *N. tabacum*×*N. gossei*. There was no direct evidence to exclude the possibility that in the absence of the *E2*, cell death was suppressed, but the homolog of this gene in GTH4S might compensate for the intrinsic function of the missing locus. This provides for another possibility, that the inhibition of cell death was not directly due to an absence of E2 *per se* from the genome of GTH4S, but rather, caused by the deletion of the neighboring region of the chromosome around this locus.

Tezuka and Marubashi (2006) suggested that the Q-chromosome which belongs to the S subgenome in *N. tabacum* was a counterpart for the expression of lethality in the hybrid of *N. tabacum*×*N. suaveolens*. Since *N. gossei* belongs to the same taxonomical section, *suaveolenstes*, *as N. suaveolens*, it is likely that the counterpart in *N. tabacum* for hybrid lethality in *N. gossei*×*N. tabacum* is located in the S subgenome. In the present study, however, we found that deletion of part of the T subgenome suppressed cell death in GTH4S. This simply means that the region missing from GTH4S contains genetic factors required for cell death reactions. There are many genes involved in the signaling pathway of programmed cell death in plants (Lam 2004), some of which function as positive regulators of cell death, eg. LOL1 in Arabidopsis. Despite this, it is also possible that the region missing from the genome of GTH4S contains problem genes which play a role in the expression of lethality in the hybrid of *N. gossei*×*N. tabacum*. The present results indicate that GTH4S is a mutant, deleted of some of the genetic components in GTH4. Sequencing of chromosomal regions around the locus of *E2* is proceeding with the screening of a genomic library of GTH4.

While mass spectrometry in combination with two-dimensional gel electrophoresis has become a primary method for the identification of proteins, several alternative approaches are available to separate complex mixtures. To find proteins that might be involved in the expression of cell death, total soluble protein extracted from GTH4 and GTH4S cells was analyzed with anion exchange chromatography and SDS-PAGE. Both cell lines were cultured at 37°C. The cells were homogenized with 50 mM Tris-HCl buffer (pH 7.6) containing 0.1% (v/v) 2-mercaptoethanol under ice-cold conditions, and centrifuged at 20,397×*g* for 15 min. The supernatant was used as the protein sample. The proteins were adsorbed onto a wide-pore DEAM Column (particle size 5μm, column size 4.6×50 mm, J.T. Baker) for HPLC (LC-10Ai, SHIMADZU), and eluted with 50 mM Tris-HCl buffer (pH 8.0) containing NaCl (gradient from 0 to 0.5 M). One milliliter of elution was collected from fraction no.1 to no. 40. Samples of each fraction from GTH4 or GTH4S were separated side by side by SDS-PAGE, and protein bands were detected using a Silver Staining Kit (Wako Pure Chemical Co.). The profile of anion-exchange chromatography was similar between GTH4 and GTH4S, suggesting that the composition of proteins of the two cell lines did not differ (Figure 4A). SDS-PAGE revealed this clearly (Figure 4B): we found 3

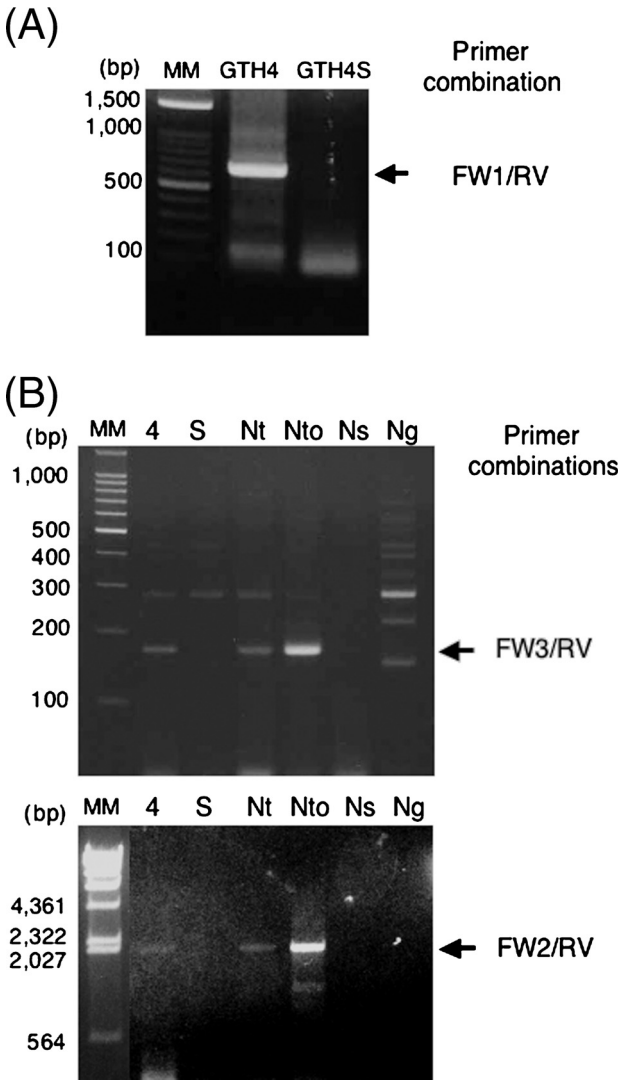

Figure 4. Detection of *E2*. (A) *E2* is expressed in GTH4, but not in GTH4S. RT-PCR was conducted with total RNA isolated using the primers FW1 and RV2 shown in Figure 3A, which were designed using the 5′ and 3′ UTR, respectively, for the specific detection of *E2*. The expected 655-bp PCR product is indicated by an arrow. (B) Top panel; *E2* is present in the genome of GTH4 (4), *N. tabacum* (Nt), and *N. tomentosiformis* (Nto), but not GTH4S (S), *N. sylvestrism* (Ns), or *N. gossei* (Ng). The expected 168-bp product of the PCR targeting 3′ UTR with the primers FW3 and RV is indicated by an arrow. Bottom panel; The 2-kbp PCR product indicated by an arrow was detected using the primers FW2 and RV, suggesting the presence of a long intron in the genomic DNA between the sites of two primers. Fifty nanograms of DNA prepared by CTAB methods (Rogers and Bendich 1994) was used as a template. MM: molecular marker. All bands otherwise indicated by arrows are nonspecific PCR products.

and 2 protein bands which were specific to GTH4 and GHT4S, respectively, while no apparent differences were found in the rest of the bands between the two cell lines. The protein samples which contained bands specific to GTH4 were concentrated using an Ultrafree-CL Filter (MILLIPORE), subjected to SDS-PAGE, and transferred onto PVDF membranes via electroblotting. The proteins were visualized by Coomassie staining. The corresponding protein was directly applied to a protein sequencer (PPSQ-21, Shimadzu) for determination of the N-terminal sequence. The partial amino acid sequence determined in a sample was analyzed using the Swiss-Prot Protein database. This protein had weak homology to a putative adenosine-5′-phosphosulfate (APS) reductase (Q56X39) of Arabidopsis (white arrowheads of fraction number 14 in Figure 5B). The APS reductase is a key enzyme of sulphate reduction in plants, and the activity of this enzyme is induced by many forms of abiotic stress (Hesse et al. 2003). To conduct more precise experiments, we will purify larger amounts of proteins which are specific to GTH4.

In conclusion, the results from our study on cultured cells of the lines GTH4 and GTH4S allow us to address the identification of hybrid problem genes or proteins. Since GTH4S is a deletion mutant of GTH4, revealing the genetic factors missing from the genome of this cell line may provide clues for the study of speciation. Although genetic analyses are a powerful tool for identifying hybrid problem genes, analyses of expressed genes and proteins are useful for the study of hybrid lethality in recalcitrant interspecific hybrid plants.

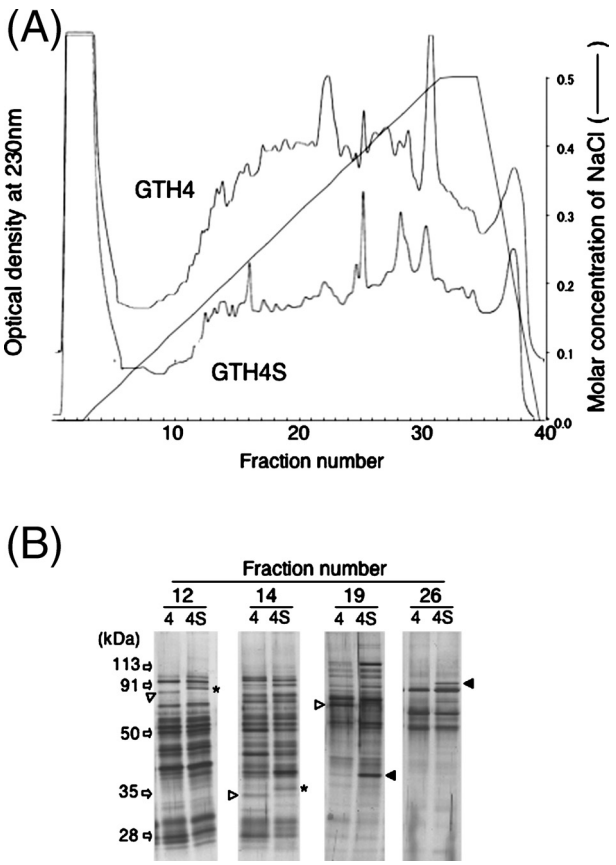

Figure 5. Separation of total soluble protein by anion exchange HPLC and SDS-PAGE. (A) Comparison of chromatograms of the proteins from GTH4 and GTH4S. (B) Each fraction was loaded on the gel, and proteins were visualized by silver staining. The fractions showing different protein profiles between GTH4 and GTH4S are indicated. The numbers on the top of the panel correspond to these at the bottom of the panel in (A). White and black arrowheads indicate the protein band specific to GH4 and GTH4S, respectively. The protein bands marked with a white arrowhead in fraction 14 showed homology to a putative adenosine-5′-phosphosulfate (APS) reductase (Q56X39) of *Arabidopsis thaliana* (see text). The protein bands marked with asterisks in fractions 12 and 14 are not specific to GTH4S, because they appeared in the next fraction of GTH4.

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