Evolution of a Basic Helix-Loop-Helix Protein from a Transcriptional Repressor to a Plastid-resident Regulatory Factor

INVOLVEMENT IN HYPERSENSITIVE CELL DEATH IN TOBACCO PLANTS

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The tobacco gene NtWIN4 (Nicotiana tabacum wound-induced clone 4) is transiently up-regulated in response not only to wounding but also to pathogen attack. NtWIN4 encodes a putative basic helix-loop-helix protein with an apparent molecular mass of 28 kDa that exhibited clear nuclear transcription repression activity in Dual-Luciferase assays. However, immunoblotting indicated the existence of a 17-kDa form of NtWIN4 localized exclusively in tobacco leaf chloroplasts. Subsequent peptide dissection analyses with green fluorescent protein fusions revealed that a polypeptide of 81 amino acids starting at position 13 from the N terminus is maximally necessary for this localization. Further fine dissection analysis strongly suggested that the protein actually begins at the second Met located at position 27, yielding a signal peptide of 67 amino acids. However, the last C-terminal 15 amino acids overlap with the conserved basic region critical for DNA binding, so NtWIN4 presumably does not function as a transcription factor in planta. Transgenic tobacco plants constitutively over-expressing NtWIN4 demonstrated mortality with abnormal features, including albinism, and transient expression upon agroinfiltration resulted in distinct necrosis with a sharp decrease in chlorophyll content, consistent with the phenomenon known as chlorosis. Transgenic RNA interference tobacco plants exhibited reduced hypersensitive cell death, showing delayed tissue necrosis upon pathogen infection. These results suggest that NtWIN4 arose by divergence, becoming a chloroplast-resident factor from a nuclear transcriptional repressor by obtaining a transit peptide sequence, and that, upon translocation, it interacts with chloroplast components to induce hypersensitive cell death through chloroplast disruption, thereby contributing to plant stress responses.

Higher plants are constantly exposed to diverse environmental stresses, including physical wounding. Under natural conditions, the major cause is grazing by herbivores, which continuously damages tissues, occasionally resulting in whole plant death after secondary infection by pathogens. To cope with this problem, plants have evolved a variety of self-defense mechanisms, including production of proteins such as proteinase inhibitors and of signaling components such as jasmonate and systemin (1–3). Wound signals are quickly transmitted from damaged cells to the whole plant, allowing elaboration of a defense reaction by transcriptionally up- and/or down-regulating a number of genes (4). Examples are proteinase inhibitors against insect feeding, extensions for reinforcement of cell walls, phenylalanine ammonia-lyase (EC 4.3.1.5) and chalcone synthase (EC 2.3.1.74) for phenylpropanoid synthesis, and 1-aminocyclopropane-1-carboxylate synthase and aminocyclopropane-1-carboxylate oxidase for ethylene synthesis (1, 5, 6). However, their functions have not been completely clarified.

One of the phenotypic hallmarks of wounding is tissue necrosis. Cells at damaged areas rapidly die, thereby forming a physical barrier to secondary injury such as pathogen attack. Biochemically, chlorophyll degradation is one characteristic symptom of wounding, this being called chlorosis (7). Recent studies have shown that this occurs not only by wounding but also during pathogen responses, suggesting that chlorophyll degradation is a common response in stressed plants. For example, the photosystem II complex was reported to be disrupted during the hypersensitive response upon inoculation of tobacco mosaic virus (TMV) (8). Accumulation of TMV coat proteins in chloroplasts has been suggested to correlate with severe chlorosis (8). Another example of a chloroplast protein involved in the hypersensitive response is chloroplast-resident FtsH, a member of the AAA group of proteins, the reduction of which results in acceleration of the hypersensitive response in tobacco plants (9). These observations indicate a close correlation between disruption of chloroplast function, including chlorosis, and the hypersensitive response, although the underlying molecular mechanisms remain to be determined.

The basic helix-loop-helix (bHLH) proteins form a superfamily of nuclear transcription factors characterized by the conserved basic region (10, 11). bHLH transcription factors have been shown to regulate many developmental and stress-responsive processes in plants (12–14). However, their involvement in the hypersensitive response remained poorly understood.

The abbreviations used are: TMV, tobacco mosaic virus; bHLH, basic helix-loop-helix; RACE, rapid amplification of cDNA ends; RT, reverse transcription; Gal4BD, Gal4 DNA-binding domain; CaMV, cauliflower mosaic virus; GFP, green fluorescent protein; MES, 4-morpholineethanesulfonic acid.
Functional Diversification of bHLH Protein

presence of a bHLH domain, a motif of ∼60 amino acids with two functional regions involved in DNA binding and dimerization (10, 11). The basic region located at the N-terminal end of the domain binds to a consensus hexanucleotide E box (CANNTG), and the helix-loop-helix region at the C-terminal end of the domain functions to form homo- and/or hetero-dimers (10, 11). In plants, the maize LC protein is the first reported bHLH family member and is involved in regulation of anthocyanin synthesis (12). Subsequent analyses of the Arabidopsis genome identified 147 genes encoding bHLH proteins, so this constitutes the largest transcription factor family (13, 14). The members are considered to be important regulatory factors in nuclear transcriptional networks, like their mammalian counterparts. However, the exact biological functions of the majority of plant bHLH proteins remain to be determined.

During the past decade, we have screened genes for which transcripts accumulate immediately after wound stress by fluorescence differential display and have so far isolated >50 clones (15). Although their identities are diverse, the majority of transcripts were found to accumulate within 1 h of mechanical injury, suggesting critical roles in defense responses. In this study, one particular clone was subjected to further characterization because of its specific feature of encoding a novel bHLH protein localized in chloroplasts and contributing to the hypersensitive response through chlorosis.

EXPERIMENTAL PROCEDURES

Plant Materials and Treatment—Tobacco plants (Nicotiana tabacum cv. Xanthi NC) were grown in soil in a growth cabinet at 23 °C under a 14-h light/10-h dark photo cycle. Protoplasts of the above-described plants and tobacco BY2 (Bright Yellow 2) and Arabidopsis MM2d cells were maintained continuously in the dark at 23 °C in suspension culture using modified Linsmaier and Skoog medium (16). Wound stress was applied by cutting mature leaves of N. tabacum with a pair of scissors or a leaf punch. Wounded (local) and adjacent upper unwounded (systemic) leaves were harvested at appropriate time points. For TMV infection, leaves of 2-month-old wild-type plants were inoculated with TMV (10 μg/ml) and incubated at 30 °C under continuous light for 48 h and then at 20 °C (temperature shift) as described previously (17). Samples were harvested at appropriate time points. For treatment with stress-signaling molecules, leaf disc samples were transferred into a solution containing 500 μM salicylic acid, 50 μM methyljasmonic acid, 100 μM ethephon (for ethylene production), or 10 mM hydrogen peroxide and harvested after 12 h. For time course analyses, healthy leaves were treated with 50 μM methyljasmonic acid or 100 μM paraquat and harvested at appropriate time points. Bacterial infection was performed as reported previously (18) with inoculation of Pseudomonas syringae by infiltration, followed by observation after 48 h.

Isolation of NtWIN4 cDNA—A DNA fragment encoding NtWIN4 was initially obtained from a cDNA population derived from wounded tobacco leaves by fluorescence differential display (15). The full-length cDNA sequence was successfully isolated by 5′-rapid amplification of cDNA ends (RACE) using primer 5′-AGAAGAGACGAGGTCTTCTTCCAG-3′ and a Marathon cDNA amplification kit (Clontech) and by the thermal asymmetric interlaced PCR method (19) using three thermal asymmetric interlaced primers (5′-NGTGGASWGAANAWG-3′, 5′-GTNGASWCAANAWGT-3′, and 5′-WGTGNAWGANAGA-3′) and three NtWIN4 primers (5′-CAATTGGGAGCATTTATAGAATATCAAATT-3′, 5′-CTCCATCATCTCTTGTTCTGTCCATGGTCG-3′, and 5′-TCGCGGATACTCACCTTGGGGACGAGAATCTC-3′). The nucleotide sequence of the isolated DNA was determined for both strands with a BigDye terminator sequencing kit (Applied Biosystems). The 5′ terminus was determined by 5′-RACE with primer 5′-ATGATTTCGCGGATCATTCTCCA-3′ (nucleotides 161–182 from the 5′-end).

DNA and RNA Analyses—Genomic DNA was isolated from green leaves by the cetyltrimethylammonium bromide method (20) with modifications, and a 20-μg aliquot was digested with one of a series of restriction enzymes: BamHI, EcoRV, or HindIII. Total RNA was isolated by the aurotricarboxylic acid method (21), and DNA and RNA gel blot hybridization was performed as described previously (22) using an NtWIN4 cDNA fragment as the probe. For PCR and reverse transcription (RT)–PCR analyses, NtWIN4 genomic and cDNA fragments were amplified by PCR with Ex Tag DNA polymerase (Takara, Ohtsu, Japan) using primers 5′-GCTCTCAGATGAAATCAAATGGTTTTTGTCG-3′ (forward) and 5′-TACCACATGGGTTCGTTCTGCTGCTGAAG-3′ (reverse).

Protein Analyses—Tobacco leaves were ground in liquid nitrogen in a mortar; mixed with isolation buffer containing 50 mm Tris-HCl (pH 7.5), 100 mM NaCl, 0.05% (w/v) Tween 20, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin; and homogenized. After centrifugation at 15,000 rpm for 10 min to remove insoluble debris, crude extracts were directly used for Western blot hybridization. After fractionation by SDS-PAGE, proteins were electronically transferred onto cellulose membranes (Immobilon-N, Millipore, Bedford, MA), and NtWIN4 was detected with rabbit antibodies raised against a synthetic peptide (MIKERRREKQOSY) and horseradish peroxidase-conjugated anti-rabbit antibodies (Bio-Rad). Fractionation of cytoplasmic, mitochondrial, and chloroplast fractions was performed as described (23–26).

Nuclear Transcription Assays—The Gal4BD-NtWIN4 and Gal4BD-At5g43650 effector plasmids were constructed by fusing a cDNA encoding NtWIN4 or At5g43650, respectively, with the Gal4 DNA-binding domain (Gal4BD) in a yy64 vector, a derivative of pMA560 (27). NtWIN4 and At5g43650 were thus subcloned into the BglII and Sall sites of yy64, respectively. As an effector control, the yy64 vector alone was used. The reporter plasmid (yy96) contains a luciferase gene placed under the control of the Gal4-binding site (27). An internal control plasmid containing a Renilla luciferase gene placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter was used to normalize for differences in bombardment efficiency. Five-day-old BY2 and MM2d cells were plated on 1/2 Murashige-Skoog agar and bombarded with plasmids (effector/reporter/reference = 2:2:1) coated on a 1.0-μm microcarrier under a vacuum of 28 inches of mercury using a helium pressure of 1100 p.s.i. (PDS-1000, Bio-Rad). Cells were placed 6 cm from the stopping screen. After bombardment, they were incubated in the dark at 28 °C for 24 h. Luciferase and Renilla luciferase activities were assayed using a Dual-Luciferase 3 K. Hara, C. Ueda, H. Ohya, and H. Sano, unpublished data.
Epifluorescence Analyses—Full-length or partial cDNA regions of NtWIN4 were subcloned into the SalI/NcoI sites of a CaMV 35S-sGFP(S65T)-NOS vector, harboring a synthetic gene for improved green fluorescent protein (GFP) S65T driven by the CaMV 35S promoter and the NOS terminator (28). Onion epidermal cell layers were bombarded with gold particles (Bio-Rad) coated with appropriate vector constructs; and after incubation at 28 °C for 6–12 h in the dark, samples were viewed under a microscope (Olympus Provis AX70 and Leica MZ FLIII) equipped with a fluorescence module.

Transgenic and Agroinfiltration Analyses—A cDNA fragment encoding NtWIN4-(27–247) was amplified by PCR and subcloned into the BamHI/SacI sites of pBI121 (Clontech). The resulting plasmid, pBI-NtWIN4-(27–247), was transformed into Agrobacterium tumefaciens strain LBA4404 (29) and used for stable transformation of tobacco as described previously (30). For agroinfiltration, A. tumefaciens LBA4404 transformed with pBI-NtWIN4-(27–247) was grown on an LB agar plate for 2 days in the dark and suspended in a solution containing 10 mM MES-NaOH (pH 5.5) and 200 μM acetosyringone to give an absorbance of 0.5 at 600 nm. The bacterial solution was directly infiltrated into a leaf segment as described (31). The chlorophyll contents in infiltrated regions were measured using a SPAD-502 chlorophyll meter (Minolta, Tokyo, Japan). Chlorophyll fluorescence was measured with a MINI-PAM pulse amplitude-modulated fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The minimal chlorophyll fluorescence at the open photosystem II center ($F_o$) was determined by measuring light at a light intensity of 0.05–0.1 μmol photons m$^{-2}$ s$^{-1}$. A saturating pulse of white light (800 ms, 8000 μmol photons m$^{-2}$ s$^{-1}$) was applied to determine the maximal chlorophyll fluorescence at the closed photosystem II center in the dark ($F_m$). The maximal quantum yield of photosystem II was calculated by the following equation: $(F_m - F_o)/F_m$.

RESULTS

Isolation of NtWIN4—One clone of >50 genes identified by fluorescence differential display activated immediately after mechanical wounding in tobacco leaves (15), tentatively named NtWIN4 (Nicotiana tabacum wound-induced clone 4), was further characterized in this study. Determination of its full-length sequence by 5′-RACE yielded a 949-bp cDNA. NtWIN4 transcripts transiently accumulated only in local leaves within 30 min after wounding, reaching maximal levels at 1 h and declining thereafter (Fig. 1A). RT-PCR indicated NtWIN4 to be constitutively expressed at a low level even in unwounded leaves (Fig. 1B), suggesting a basic function. The copy number of NtWIN4 in the genome of N. tabacum was estimated by Southern blot analysis by digesting genomic DNA with BamHI or HindIII, for which sites

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Identification of NtWIN4. A and B, time course analyses of NtWIN4 transcript accumulation. Total RNA was isolated from wounded (local (L)) and unwounded (systemic (S)) leaves at the indicated time points after wounding. A 10-μg aliquot was fractionated per lane on a 0.8% agarose gel and subjected to RNA blot hybridization with the $^{32}$P-labeled NtWIN4 cDNA probe. As the loading control, rRNA was applied (A). Transcript accumulation was also examined by RT-PCR. NtWIN4 was amplified by 35- or 25-cycle PCR. As internal controls, 18 S rRNA and actin were amplified for 25 cycles (B). C, DNA blot hybridization. A 10-μg aliquot of total DNA was digested with the indicated restriction enzymes and subjected to hybridization with the $^{32}$P-labeled NtWIN4 cDNA probe. The sizes of the DNA are indicated on the left.
FIGURE 2. Properties of NtWIN4. A, comparison of amino acid sequences. Alignment of NtWIN4, At5g43650 (A. thaliana), and OJ1365_D05.18 (Oryza sativa) was performed with the ClustalW program. Arrows indicate the bHLH motif, and identical residues shared by at least two samples are boxed. B, schematic illustration of plasmids used in the bHLH-mediated transcription assay. NosP, nopaline synthase promoter; NosT, nopaline synthase terminator; GAL4UAS, Gal4 upstream activating sequence; 35S mini, CaMV 35S minimal promoter; 35S, CaMV 35S promoter. C, transcription activity assay results. Effector constructs encoding Gal4BD-NtWIN4 or Gal4BD-At5g43650 were co-bombarded with the reporter and reference plasmids into 7-day-old BY2 or MM2d cells, respectively. Gal4BD was applied as the effector control. The luciferase (Luc) activity of each type of transformant was normalized to the respective Renilla luciferase (R-Luc) activity. The relative luciferase activity was calculated by dividing the luciferase activity of each clone by that of the clone containing the Gal4BD effector construct (triplicate experiments).
**Functional Diversification of bHLH Protein**

**Transcription Repression Activity**—Full-length NtWIN4 was found to encode a protein of 247 amino acids with an apparent molecular mass of 28.7 kDa (Fig. 2A). A database search demonstrated possession of a bHLH motif, characteristic of a transcription factor. A PHI-BLAST homology search indicated close similarity in the bHLH motif and C-terminal region to proteins from *Arabidopsis* (At5g43650) and rice (OsJ1365_D05) (Fig. 2A). To test whether or not the NtWIN4 and At5g43650 proteins exhibit transcriptional activity, nuclear transcription assays were carried out in vivo by the Dual-Luciferase assay method. Effector constructs of Gal4BD-NtWIN4 and Gal4BD-At5g43650 were co-bombarded with a reporter plasmid and a reference plasmid into tobacco BY2 and *Arabidopsis* MM2d cells, respectively. The results showed that the Gal4BD-NtWIN4 and Gal4BD-At5g43650 effector constructs suppressed luciferase activity to a level one-third that of the Gal4BD control (Fig. 2C). The results indicate that the NtWIN4 and At5g43650 proteins possess transcription repression activity in the nucleus, suggesting that their structures confer common functions.

**Cellular Localization**—To confirm nuclear localization, GFP was fused to the C terminus of NtWIN4 or At5g43650 and assayed in onion epidermal cell layers after particle bombardment. GFP fluorescence from both NtWIN4-GFP and At5g43650-GFP resembled that from the GFP-only control, suggesting both to be localized in the cytoplasm and/or nucleus (Fig. 3A). To

**FIGURE 3. Cellular localization.** A, localization of full-length NtWIN4. NtWIN4-(1–247)-GFP and At5g43650-(1–247)-GFP fusion proteins were transiently expressed in onion epidermal cells (left and middle panels, respectively). GFP alone was used as a control (right panel). B, identification of NtWIN4 protein. Crude extracts of wounded tobacco leaves were fractionated by SDS-PAGE and stained with Coomassie Brilliant Blue (CBB) (left panel) and then subjected to immunoblot hybridization using an antibody raised against an NtWIN4 peptide (right panel). Protein size markers were fractionated in parallel (lane 1). The positions of the large (RBCL) and small (RBCS) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase are indicated by arrows. The arrowhead indicates NtWIN4. C, time course analysis of NtWIN4 protein accumulation. Healthy leaves were wounded and harvested at the indicated time points to extract crude proteins, which were fractionated by SDS-PAGE and subjected to immunoblot hybridization. The positions of the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase are indicated by arrows and NtWIN4 is indicated by the arrowhead. Equal loading of proteins was monitored with the large subunit stained with Coomassie Brilliant Blue. D, fractionation of cellular components. Crude extracts were subjected to gradient centrifugation, and isolated cytoplasmic, mitochondrial, and chloroplast fractions were fractionated by SDS-PAGE and subjected to immunoblot hybridization. The positions of the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase are indicated by arrows, and NtWIN4 is indicated by the arrowhead. Equal loading of proteins was monitored with the large subunit stained with Coomassie Brilliant Blue. E, purity verification. Total cell and chloroplast fractions were analyzed by immunoblot hybridization using antibodies against compartment-specific proteins: the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (chloroplast), DRM (nucleus), and phosphoenolpyruvate carboxylase (PEPC, cytoplasm). F, plastid localization of NtWIN4-(27–247) and At5g43650-(31–114). Upper panel, 47 amino acids at the N termini of NtWIN4 and At5g43650 are shown. The second Met at position 27 in NtWIN4 (closed arrowhead) and Val at position 30 in At5g43650 (open arrowhead) are indicated. Lower panels, onion epidermal cell layers were bombarded with NtWIN4-(27–247)-GFP or At5g43650-(31–114)-GFP, and fluorescence was observed. The controls used were AtCYS1-GFP (mitochondria) (63), chloroplast ACS1 (cpACS1)-GFP (plastid) (64), yellow fluorescent protein (YFP)-SKL (peroxisome) (65), and yellow fluorescent protein alone.
verify this result, the exact localization of NtWIN4 was biochemically examined using antibodies raised against synthetic peptides. First, total proteins were extracted from tobacco leaf tissues, fractionated by SDS-PAGE, and subjected to immunoblot staining. A clear single signal was found at the 17 kDa, suggesting the native form of NtWIN4 to be 17 kDa (Fig. 3B). This was confirmed by an increase in the 17-kDa fraction upon wounding of leaf tissues, showing maximal levels after 1 h (Fig. 3C), the pattern being consistent with that of transcript accumulation (Fig. 1A). Second, cellular components were fractionated and subjected to immunoblot analysis. When the cytoplasm, mitochondria, and chloroplasts were mechanically separated and analyzed with antibodies, 17-kDa NtWIN4 was detected in fractions containing total proteins and chloroplasts, but not in cytoplasmic and mitochondrial fractions (Fig. 3D). The chloroplast fraction was further examined for purity with control antibodies against compartment-specific proteins: the chloroplast-resident large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase and nucleus-specific DRM (domains-rearranged methyltransferase) (32) and cytoplasm-specific phosphoenolpyruvate carboxylase. It was evident that the chloroplast fraction did not contain any contaminating nuclear fractions (Fig. 3F). These results showed native NtWIN4 to be 17 kDa and exclusively localized in chloroplasts. No molecular species of 28-kDa NtWIN4 was detectable in any fraction of tobacco plants; and therefore, GFP analyses with an artificially constructed full-length 28-kDa protein (Fig. 3A) could not show the exact localization of native NtWIN4. However, because the computer-predicted molecular mass of NtWIN4 is 28.7 kDa, processing must occur in planta, facilitating movement into chloroplasts. Subsequently, the N terminus of NtWIN4 was successively truncated to identify the signal peptide for chloroplast import by GFP assay. When beginning at the second Met at position 27, the protein was distinctly incorporated into plastids, but not into mitochondria or peroxisomes (Fig. 3F). Thus, the chloroplast transit signal is located in peptides lacking the first 26 amino acids; and therefore, additional analyses were performed with the protein beginning at the second Met, NtWIN4-(27–247) (Table 1). To determine whether the Arabidopsis counterpart contains such a transit signal, a mutant protein lacking the first 30 amino acids (At5g43650-(31–114)) was constructed for GFP fusion, although the native At5g43650 protein does not possess the second Met at the position corresponding to NtWIN4. The results clearly showed At5g43650-(31–114) to be localized in plastids (Fig. 3F), suggesting that amino acid sequences that are potentially able to serve as signals for plastid localization are conserved among NtWIN4 ortholog proteins.

**Transit Peptides for Chloroplast Localization**—No apparent chloroplast transit signal peptide in NtWIN4 and NtWIN4-(27–247) was predicted from *in silico* analysis with the transit peptide prediction server ChloroP 1.1 (www.cbs.dtu.dk/services/ChloroP/) or the localization prediction servers TargetP 1.1 (www.cbs.dtu.dk/services/TargetP/) and WoLF PSORT (wolfsort.seq.cbr.jrc.it/) (33–35). However, when sequences in the N-terminal region (from position 27 to between positions 73 and 148) were subjected to prediction by TargetP 1.1, a putative chloroplast transit peptide was identified (data not shown). Similarly, the N-terminal region of At5g43650 (from position 42 for the second Met to between positions 97 and 150) was found to contain a possible transit peptide, which showed a quite high (but below the threshold) probability of chloroplast localization (data not shown). Hence, it was conceivable that these proteins originally possessed classical but diversified chloroplast transit peptides. To identify the N-terminal signal sequence, truncated N-terminal regions were fused to the N terminus of GFP (Table 1) and transiently expressed in onion epidermal cell layers by particle bombardment. The results showed that NtWIN4-(27–78)-GFP localized in the cytoplasm, whereas NtWIN4-(27–111)-GFP and NtWIN4-(27–135)-GFP clearly localized in plastids (Fig. 4A). The region between amino acids 78 and 111 contains the basic region and the first helix domain of the bHLH motif, suggesting a critical role. Subsequently, a fine dissection assay was carried out within these regions. NtWIN4-(27–91)-GFP distinctly localized in plastids, whereas NtWIN4-(27–93)-GFP clearly localized in plastids (Fig. 4B). This indicates that eight amino acids between positions 87 and 95 are critical for signaling. Additional assays revealed NtWIN4-(27–91)-GFP and NtWIN4-(27–92)-GFP in the cytoplasm, but NtWIN4-(27–93)-GFP and NtWIN4-(27–94)-GFP in plastids (Fig. 4B). We thus concluded that the chloroplast localization signal is 93 amino acids from the N terminus of NtWIN4 (Fig. 4B and Table 1). This was confirmed with cultured tobacco cells, in which NtWIN4-(27–87)-GFP and NtWIN4-(27–95)-GFP localized to the cytoplasm and chloroplasts, respectively (Fig. 4C). The molecular mass of NtWIN4 lacking the first 93 amino acids is computer-predicted to be ~17.7 kDa, similar to the mass of the native NtWIN4 protein. These results indicate that the transit peptide extends into the basic region, which usually functions as the DNA-binding domain of the bHLH motif, and therefore suggest that native NtWIN4 might be unable to bind to DNA in chloroplasts. Because *Arabidopsis* At5g43650-(31–114)-GFP was clearly shown to localize to plast-

### TABLE 1

| Designation     | No. of residues | Cellular localization          |
|-----------------|----------------|--------------------------------|
| NtWIN4          | 247            | Cytoplasm and nucleus          |
| NtWIN4-(27–78)  | 52             | Cytoplasm and nucleus          |
| NtWIN4-(27–87)  | 61             | Cytoplasm and nucleus          |
| NtWIN4-(27–91)  | 65             | Cytoplasm and nucleus          |
| NtWIN4-(27–92)  | 66             | Cytoplasm and nucleus          |
| NtWIN4-(27–93)  | 67             | Chloroplast                    |
| NtWIN4-(27–94)  | 68             | Chloroplast                    |
| NtWIN4-(27–95)  | 69             | Chloroplast                    |
| NtWIN4-(27–111) | 85             | Chloroplast                    |
| NtWIN4-(27–135) | 109            | Chloroplast                    |
| NtWIN4-(27–247) | 221            | Chloroplast                    |
| NtWIN4-(7–111)  | 105            | Cytoplasm and nucleus          |
| NtWIN4-(8–111)  | 104            | Cytoplasm and nucleus          |
| NtWIN4-(9–111)  | 103            | Cytoplasm and nucleus          |
| NtWIN4-(10–111) | 102            | Cytoplasm and nucleus          |
| NtWIN4-(11–111) | 101            | Cytoplasm and nucleus          |
| NtWIN4-(12–111) | 100            | Cytoplasm and nucleus          |
| NtWIN4-(13–111) | 99             | Chloroplast                    |
| NtWIN4-(14–111) | 98             | Chloroplast                    |
| NtWIN4-(21–111) | 91             | Chloroplast                    |
| NtWIN4-(31–111) | 81             | Chloroplast                    |
| NtWIN4-(36–111) | 76             | Chloroplast                    |
| NtWIN4-(37–111) | 75             | Chloroplast                    |
| NtWIN4-(38–111) | 74             | Chloroplast                    |
| NtWIN4-(39–111) | 73             | Chloroplast                    |
| NtWIN4-(40–111) | 72             | Cytoplasm and nucleus          |

35374 JOURNAL OF BIOLOGICAL CHEMISTRY


**Functional Diversification of bHLH Protein**

The above observations implied that native NtWIN4 does not function as a nuclear transcriptional regulator. The question then arises as to its biological role in chloroplasts. NtWIN4 was initially identified as a wound-induced gene (Fig. 1A), suggesting a contribution to stress responses. Subsequently, transcript induction by stress-signaling molecules was examined by treating leaves of wild-type tobacco plants with salicylic acid, methyljasmonic acid, ethylene (for ethylene production), or hydrogen peroxide. RT-PCR showed that the transcripts were apparently induced by methyljasmonic acid and hydrogen peroxide (Fig. 6A), and time course analyses confirmed this, exhibiting transient accumulation with methyljasmonic acid at 3 h (Fig. 6B) and with paraquat (a hydrogen peroxide generator) (Fig. 6C) between 1 and 3 h. The latter result suggested that NtWIN4 responds to reactive oxygen species, which are generated by various stresses and which play critical roles as inducers of the defense reaction. One such activity is the induction of programmed cell death, which typically occurs during pathogen responses (17).

Accordingly, the effect of biotic stress was examined by challenging leaves with TMV. When tobacco plants carrying the resistant gene (N gene) are inoculated with TMV and incubated at 30 °C, at which temperature the N gene does not function, viral particles multiply; upon transfer to 20 °C, the N gene is activated, resulting in a resistance called the hypersensitive response (36). Results using this experimental system showed that NtWIN4 transcripts were induced 3 h after the onset of this hypersensitive response and accumulated thereafter up to 9 h.

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**FIGURE 4. Identification of a transit peptide.** A, schematic illustration of the NtWIN4-(27–247) structure. Arrows indicate the sizes of truncated NtWIN4 with GFP fused at the C terminus. Onion epidermal cell layers were bombarded with plasmids containing the indicated constructs, and fluorescence was observed. The samples used were NtWIN4-(27–95)-GFP (panel a), NtWIN4-(27–94)-GFP (panel b), NtWIN4-(27–93)-GFP (panel c), NtWIN4-(27–92)-GFP (panel d), NtWIN4-(27–91)-GFP (panel e), and NtWIN4-(27–87)-GFP (panel f). C, cultured tobacco protoplast-derived cells expressing NtWIN4-(27–95)-GFP (panels a) or NtWIN4-(27–87)-GFP (panels f), GFP fluorescence (right panels) and chlorophyll autofluorescence (middle panels) are merged (right panels).

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| 27-78 | 135 | 247 aa |
|-------|-----|--------|
| NtWIN4^{27–78} |
| NtWIN4^{27–135} |
| NtWIN4^{27–143} |
| NtWIN4^{27–247} |

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| Basic | Helix |
|-------|-------|
| MEREKVHKHMKIERIRREKOKQSYLDLHKLLP... |

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**Expression Profile**

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**Minimal Requirement for a Transit Peptide**

To determine the minimal size required to function as a transit peptide, dissection assays were carried out by constructing peptides beginning at variable positions from the N terminus and terminating at position 111 (Fig. 5A). First, regions upstream from position 27 (Met) were examined. NtWIN4-(7–111)-GFP localized in the cytoplasm, whereas NtWIN4-(14–111)-GFP and NtWIN4-(21–111)-GFP localized in plastids, suggesting the minimal peptide to be between 8 and 13 amino acids from the N terminus (Fig. 5B). Subsequent fine dissection assays showed that NtWIN4-(8–111)-GFP, NtWIN4-(9–111)-GFP, NtWIN4-(10–111)-GFP, NtWIN4-(11–111)-GFP, and NtWIN4-(12–111)-GFP localized in the cytoplasm and NtWIN4-(13–111)-GFP in plastids (Fig. 5B). Regions downstream from position 27 (Met) were then examined. NtWIN4-(31–111)-GFP and NtWIN4-(36–111)-GFP localized in plastids, whereas NtWIN4-(40–111)-GFP was again found in the cytoplasm, suggesting the minimal C terminus to be between positions 36 and 39 (Fig. 5C). Fine dissection assays within this region showed that NtWIN4-(37–111)-GFP, NtWIN4-(38–111)-GFP, and NtWIN4-(39–111)-GFP distinctly localized in plastids (Fig. 5C). These results reveal that, for function as a transit peptide, amino acids between positions 13 and 39 are a prerequisite. The second Met locates within this frame at position 27.
Functional Diversification of bHLH Protein

A

MEEFQIQSS QTNNQEGVLW FENAFLMNQS AFASFRNQSI ...

B

C

FIGURE 5. Determination of the transit peptide initiation site. A, sequence of the first 47 amino acids in the N-terminal region of NtWIN4. Dissection positions are indicated by arrowheads labeled with lowercase letters. B and C, GFP epifluorescence analysis. Onion epidermal cell layers were bombarded with plasmids containing GFP fused to the indicated fragment in A, and fluorescence was observed. Truncated samples upstream of the Met at position 27 are NtWIN4-(7–111)-GFP (panel a), NtWIN4-(14–111)-GFP (panel b), NtWIN4-(21–111)-GFP (panel c), NtWIN4-(8–111)-GFP (panel d), NtWIN4-(9–111)-GFP (panel e), NtWIN4-(10–111)-GFP (panel f), NtWIN4-(11–111)-GFP (panel g), NtWIN4-(12–111)-GFP (panel h), and NtWIN4-(13–111)-GFP (panel i). Truncated samples downstream of the Met at position 27 are NtWIN4-(31–111)-GFP (panel j), NtWIN4-(36–111)-GFP (panel k), NtWIN4-(40–111)-GFP (panel l), NtWIN4-(37–111)-GFP (panel m), NtWIN4-(38–111)-GFP (panel n), and NtWIN4-(39–111)-GFP (panel o).

These observations suggest that NtWIN4 might play a common role in the defense system against biotic and abiotic environmental stresses.

Chloroplast Disruption—To obtain a more precise idea of its role, transgenic tobacco plants were generated in which NtWIN4-(27–247) was constitutively expressed under the control of the CaMV 35S promoter. Among several transgenic lines, two lines were finally selected because of high levels of NtWIN4 production as identified by Western blot hybridization (Fig. 7A, upper panel). However, both plantlets grew slowly and abnormally (Fig. 7A, lower pane) and ultimately died at very young stages. When stained with trypan blue, many cells were found to be dead (data not shown), indicating the transgene to be highly detrimental to growth. For further analysis, NtWIN4-(27–247) was transiently expressed in tobacco leaves by agroinfiltration, which expresses the transgene in only infected regions. The \( \beta \)-glucuronidase GUS gene was used as a control. Infiltrated samples were incubated under light or dark conditions for several days, and the phenotype was observed. Under light conditions, the regions infiltrated with NtWIN4-(27–247) were apparently damaged, but the control was also injured, although the extent was less (data not shown). This could be due to light stress, additively damaging the infiltrated tissues. In the dark, however, regions in which NtWIN4 was expressed clearly showed chlorosis 5 days after infiltration and distinct tissue necrosis 7 days later (Fig. 7B). Control infiltration with GUS did not cause any damage during this time period (Fig. 7B). The relative chlorophyll contents of the affected regions were found to have decreased to less than one-half and one-third of the control contents at days 4 and 5, respectively (Fig. 7C). This resulted in a clear reduction of the maximal activity of photosystem II photochemistry, to less than one-half of the control activity 4 days after agroinfiltration (Fig. 7D). Thus, NtWIN4 appears to be involved in chloroplast disruption.

Hypersensitive Cell Death—To examine gene function further, NtWIN4-silenced transgenic tobacco lines were produced by the RNA interference method. Four lines were finally selected, among which the R11 and R131 plants, which showed almost complete silencing of NtWIN4, were used for biological assays (Fig. 8A). Because NtWIN4 transcripts were induced upon pathogen infection (Fig. 6D), the effects of silencing on necrotic lesion formation were first examined. When leaves were inoculated with \( P. syringae \) pv. glycinea 801, which causes hypersensitive response-like cell death in tobacco plants (18), necrotic cell death occurred within 48 h in the controls, but not in the R11 plants, which had live tissues within the inoculated areas (Fig. 8B). Similarly, when leaves from wild-type control plants were inoculated with TMV, lesions began to develop 24 h after the temperature shift and reached the final mature necrotic stage 36 h later (Fig. 8C). In the R11 transgenic line, lesions were visible at 24 h, but did not develop to the final stage even after 48 h, with dead tissues only around the lesion, forming ring-shaped spots (Fig. 8C) (37). Direct measurement of cell death by chloroplast autofluorescence indicated that, in the R131 and R71 transgenic plants, the hypersensitive response was much delayed in comparison with wild-type controls (Fig. 8D). Taking these results overall, it is conceivable that one NtWIN4 function might be to enhance hypersensitive cell death possibly through disruption of chloroplast activity.
reveal two novel features of protein function: first, that a protein can be flexibly utilized for diverse objectives after intensive modification, and second, that a bHLH factor may regulate hypersensitive cell death probably through chloroplast disruption.

Conversion of bHLH Protein—Since the bHLH motif was first identified in transcription factors E12 and E47 in murine cells (10), a set of bHLH proteins has been identified in both plants and animals (38, 39). The number of corresponding genes has continuously increased, for example, to 35 in nematodes, 56 in the fly, and 147 in Arabidopsis (14, 40). The biological functions of bHLH proteins are currently considered to be due to their activities as transcription factors by forming homo- and/or heterodimers, but not all have been biochemically characterized (10, 11). The novel bHLH protein NtWIN4, identified in this study, shows particularly high similarity to the Arabidopsis At5g43650 protein. When NtWIN4 and At5g43650, each encoding a 28-kDa protein, were introduced in tobacco and Arabidopsis cells, respectively, clear transcription repression activity was observed in the nucleus with both. Thus, NtWIN4 can function as a transcription factor, as do most bHLH proteins. Nevertheless, further analysis revealed that NtWIN4 localized exclusively in chloroplasts. This raised two questions. First, how might it be translocated? Second, what is its function?

Proteins that are translocated to plastids usually possess a transit peptide of 40–50 amino acids at the N terminus (7) that is cleaved off during translocation, resulting in plastid-resident mature proteins of smaller sizes compared with the precursors. Translocation of NtWIN4 into chloroplasts appears to be such a typical case, with deletion of the first 93 amino acids if the protein is synthesized from the first AUG codon on the full-length mRNA sequence. However, for translocation into chloroplasts, fine dissection analyses revealed that the protein should begin at an amino acid between positions 13 and 39, suggesting that the first 12 amino acids interfere or mask the signal peptide activity.

Two scenarios conceivably account for this phenomenon. First, at least the first 12 amino acids are processed after translation. In this case, amino acids from the N-terminal end might be removed immediately after synthesis, resulting in a truncated polypeptide of <28 up to 24 kDa if the first 38 amino acids are processed. Although this possibility cannot be excluded, it appears of low probability, as no such protein species were detected in any cell fractions under our experimental conditions. Second, the protein is synthesized not from the first Met, but from the second Met, which corresponds to position 27. A study revealed that leaky scanning upon translation might occur when the first AUG codon on the full-length mRNA sequence. However, for translocation into chloroplasts, fine dissection analyses revealed that the protein should begin at an amino acid between positions 13 and 39, suggesting that the first 12 amino acids interfere or mask the signal peptide activity.

This study has documented evidence that a bHLH protein, originally possessing transcription repression activity in the nucleus, is converted to a regulatory factor for chlorosis after deletion of a basic region at the N terminus. The findings reveal two novel features of protein function: first, that a protein can be flexibly utilized for diverse objectives after intensive modification, and second, that a bHLH factor may regulate hypersensitive cell death probably through chloroplast disruption.

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that, in NtWIN4, translation starts not at the first AUG codon, but at the second in-frame AUG codon at position 27, resulting in production of a transit peptide structure composed of 67 amino acids. Taking account of these features, we came to the conclusion that NtWIN4 orthologs commonly possess potential chloroplast transit peptides and that whether or not they are active in vivo depends on the presence of preceding peptides, which usually prevent their functioning.

However, a unique feature is that this transit peptide extends into the basic region, which is considered to be the DNA-binding domain. DNA-binding proteins in chloroplasts originated mostly from prokaryotes (42); and to date, only a few have been reported with a eukaryotic origin (43–45), e.g., PD3 from pea, which binds to AT-rich sequences and might be involved in nucleoid structures (43). CND41 from cultured tobacco cells possesses both nonspecific DNA binding capacity and functional protease activity (44), whereas PEND from several species contains a basic region and a leucine zipper motif and localizes to the inner envelope membrane of developing chloroplasts. It recognizes TAA-GAAGT to bind to DNA (46) and possibly functions in linking nucleoids to the inner envelope membrane (45, 47). Amino acid sequence analyses have suggested that these proteins might originally have been nuclear transcription factors (42), providing grounds for speculation that nuclear transcription factors might have been reutilized in chloroplasts. In this context, NtWIN4 is of clear interest. Its specific feature is that, in contrast to proteins that retain DNA binding capacity, NtWIN4 has presumably lost this, so it functions solely as a novel regulatory factor in chloroplasts.

Whatever the mechanism, our findings indicate that not all bHLH proteins are nuclear transcription factors and that some could be converted to directly perform regulatory roles. Whether this feature is common among plants and/or organisms is currently not known, but it is attractive to speculate that a protein may evolve depending upon necessity.

**Biological Function**—Transgenic tobacco plants constitutively expressing NtWIN4 under the control of the CaMV 35S promoter showed abnormal phenotypes with slow growth and albinism and ultimately died before maturation. Even when NtWIN4 was spatially and temporarily expressed after agroinfiltration, the leaf color turned yellow within several days, a typical symptom of chlorosis (7). In contrast, transgenic RNA interference plants appeared to be normal under non-stressed conditions and showed delayed cell death when inoculated with pathogens (TMV and P. syrin-
amplification program, which allows detection of constitutive low level
baseline. Healthy leaves from wild-type (WT) and transgenic RNA interference
(RNAi) plants were examined for NtWIN4 expression by RT-PCR with a 35-cycle
amplification program, which allows detection of constitutive low level
expression, as shown in Fig. 1B. As an internal control, 18 S rRNA expression
was assessed. B, effects of P. syringae pv. glycinea B01 infection. Healthy leaves
from wild-type and R11 (R11) transgenic plants were inoculated with P. syringae
by infiltration and observed 48 h thereafter. In the wild-type sample,
hypersensitive response-like cell death occurred throughout the inoculated
region, whereas in the R11 sample, tissues remained alive, showing a green
color (indicated by arrows). C, effects of TMV infection. Healthy leaves from
wild-type and R11 transgenic plants were inoculated with TMV, maintained at
30 °C for 2 days, and then cultured at 20 °C for the indicated time periods.
Hypersensitive cell death was clearly apparent in the wild-type plants, with
necrotic lesions after 36 h, whereas it was not complete in the R11 plants, as
indicated by arrows. In the WT sample, tissues remained alive, showing a green
color (indicated by arrows). D, reduction of chloroplast autofluorescence. Wild-type and R11 transgenic plants were inoculated with TMV as described above, and lesions were viewed 48 h after
the temperature shift under a microscope (Olympus Provis AX70 and Leica
MZ FLIII) equipped with a fluorescence module. Because live chloroplasts
emit autofluorescence (red), they can be clearly distinguished from dead
chloroplasts in necrotic regions (yellow).

FIGURE 8. Properties of NtWIN4-silenced plants. A, confirmation of silencing. Healthy leaves from wild-type (WT) and transgenic RNA interference
(RNAi) plants were examined for NtWIN4 expression by RT-PCR with a 35-cycle
amplification program, which allows detection of constitutive low level
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Hypersensitive cell death was clearly apparent in the wild-type plants, with
necrotic lesions after 36 h, whereas it was not complete in the R11 plants, as
evidenced by live tissue inside of ring-shaped lesions. D, reduction of chloro-
plast autofluorescence. Wild-type and R11 transgenic plants were inoculated with TMV as described above, and lesions were viewed 48 h after
the temperature shift under a microscope (Olympus Provis AX70 and Leica
MZ FLIII) equipped with a fluorescence module. Because live chloroplasts
emit autofluorescence (red), they can be clearly distinguished from dead
chloroplasts in necrotic regions (yellow).

FIGURE 9. Nucleotide sequence of the 5'-untranslated region. The 5'-termi-
nal region of NtWIN4 was determined by 5'-RACE, and that of At5g3650 is
from the NCBI data base (accession number AY0653390). The first AUG codon
is boxed, and the 5'-untranslated region is underlined, with the number of nucleotides indicated: 17 for NtWIN4 and 38 for At5g3650.

Functional Diversification of bHLH Protein

hydrolysis, EC 3.1.1.14), which catalyzes the ester bond
hydrolysis of chlorophyll to yield chlorophyllide and phytol
(49, 50). Encoding genes have been isolated from multiple
plant species (51–54), among which AtCLH1 from Arabi-
dopsis is best characterized (52). AtCLH1 is up-regulated by
wounding, methyl jasmonic acids, and coronatine (a chloro-
sis-inducing phytotoxin produced by various plant patho-
genic bacteria), and the encoded protein functions not only
in defense but also during development and senescence (50,
52, 55–57). A recent study identified two additional enzymes, pheophorbide a oxygenase and red chlorophyll
catabolite reductase (both involved in light-dependent cell
death), that catalyze the breakdown of phototoxic pigments
produced during chloroplast disruption (57). Despite
detailed biochemical studies, the molecular mechanisms of
chlorosis and regulation of chlorophyll breakdown are still
poorly understood (57). In this study, we have shown that
NtWIN4 is up-regulated by woundind, methyl jasmonic acid,
and pathogens, the pattern being similar to that of AtCLH1,
and that the resultant overexpression is lethal because of
chloroplast decay. Considering these results together with
other available information, we speculate that NtWIN4
functions as a novel chlorosis-inducing factor in response to
wounding and pathogen attack, thereby enhancing cell
death, which is a prerequisite for coping with such stresses.
NtWIN4 does not possess any enzyme domains, suggesting it to
be functional in combination with other protein factors. Because it
loses the basic region or DNA-binding domain during chloroplast
translocation, it may be active as an helix-loop-helix protein, like
protein Id (inhibitor of DNA binding) and extramacrochaete (58–
60), which may act as antagonists of other bHLH proteins by form-
ing nonfunctional heterodimer complexes (61). However, chloro-
plast-resident bHLH proteins have yet to be identified in plants,
making it improbable that NtWIN4 forms heterodimers. Never-
theless, structural analyses suggested that NtWIN4 possesses a
high potential for dimerization and/or trimerization (multicoil.
ls.mit.edu/cgi-bin/multicoil)(62), affording a capacity to inter-
act with protein(s) other than bHLH in chloroplasts. Identifica-
tion of such proteins may provide clear-cut information as to its
biological roles.

Our work has indicated that a bHLH protein has changed its
functional Diversification of bHLH Protein
Functional Diversification of bHLH Protein

chloroplasts, NtWIN4 perhaps then activated chlorosis by chance, which fitted with survival of tobacco plants. Thus, our finding provides a good example of protein evolution, realized by maximal use of the limited genomic information, thereby conferring on plants the best features adapting them to diverse environmental conditions.

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