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Note

Characterization of a Loss-of-Function Mutant of Gibberellin Biosynthetic Gene *LsGA3ox1* in Lettuce

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A previous study generated lettuce (*Lactuca sativa*) mutant lines tagged by retrotransposon *Tnt1* from tobacco (*Nicotiana tabacum*) and identified a homozygous mutant, Tnt6a, that exhibited severe dwarf phenotype. Here we show that *Tnt1* is inserted into the intron of gibberellin biosynthetic gene *LsGA3ox1* in Tnt6a mutants. Expression analysis suggests that *LsGA3ox1* is nearly knocked out in the Tnt6a mutants.

Key words: dwarf; gibberellin biosynthesis; lettuce; loss-of-function mutant; retrotransposon

Gibberellins (GAs) are tetracyclic diterpenoid phytohormones that regulate a wide range of plant growth processes, including seed germination, stem elongation, and flowering.[1)] Bioactive GAs are biosynthesized from geranylgeranyl diphosphate, a common prenyl precursor of diterpenes, through several steps, and all major GA biosynthetic enzyme genes have been identified, as recently reviewed by Yamaguchi.[1)] The GA biosynthetic genes are well characterized in lettuce (*Lactuca sativa* L. cv. Grand Rapids). In lettuce, GA_1 is a major bioactive GA[2,3)] that is biosynthesized from GA_{53} through successive oxidation catalyzed by GA 20- and GA 3-oxidases, both of which belong to the 2-oxoglutarate-dependent dioxygenase family (Fig. 1A). Two sets of cDNAs encoding the two oxidases have been isolated from lettuce: *LsGA20ox1*, *LsGA20ox2*, *LsGA3ox1*, and *LsGA3ox2*.[4,5)] Transcript levels of *LsGA3ox1* and *LsGA3ox2* are upregulated by phytochrome in lettuce seeds,[4,5)] resulting in an increase in the endogenous level of GA_1 after red-light pulse treatment.[3)] Upregulation of *LsGA3ox1* expression might be involved in the increase of GA_1 in lettuce bolting stems under high-temperature conditions.[6)]

A series of mutants that impair GA biosynthetic genes has been used to study the functions and physiological roles of GAs in *Arabidopsis*.[1)] In contrast, loss-of-function mutants of the GA biosynthetic genes in lettuce have not been widely used, although GA-related dwarf lettuce mutants have been reported.[7,8)] Recently, gene tagging in lettuce cv. Jessy using the *Tnt1* retrotransposon from tobacco (*Nicotiana tabacum*) was performed.[9)] *Tnt1* is an active 5.3-kb copia-like long terminal repeat retroelement from tobacco.[10)] Among a series of mutant lines of the Jessy background, Tnt6a mutants displayed a severe dwarf phenotype, owing to *Tnt1* insertion in the *LsGA3ox1* gene. Homozygous Tnt6a mutants containing one *Tnt1* copy are obtained by two-step backcross and successive self-pollination of T_3 progeny.[9)] Here we further characterize the homozygous Tnt6a mutant.

We confirmed the genotype of the Tnt6a mutants by polymerase chain reaction (PCR) using a three-primer mix consisting of GiF1, GiR3, and LTR1R (Fig. 1B), as previously described.[9)] Genomic PCR was performed using two lettuce plants of each genotype. The Tnt6a seeds used were obtained by self-pollination of the single-copy-*Tnt1* homozygous Tnt6a mutants described in a previous report.[9)] A band of the expected size was amplified from each sample (Fig. 2A), indicating that the Tnt6a lettuce mutants used were homozygous. Each band was subcloned into pGEM-T-easy vector (Promega, Madison, WI) and sequenced. We also determined the genomic DNA sequence corresponding to the *LsGA3ox1* open reading frame (ORF) in cv. Grand Rapids. Genomic PCR was performed using a primer set for ORF amplification described previously,[4)] and the nucleotide sequence of the 1,276-bp fragment was determined (AB636282). A comparison of the genomic DNA sequence with the cDNA sequence of *LsGA3ox1* (AB012205)[4)] indicated that the *LsGA3ox1* gene contained one 184-base intron (Fig. 1B). The sequence of the 847-bp fragment from wild-type (WT) cv. Jessy amplified with primers GiF1 and GiR3 was identical to that of the corresponding genomic region in cv. Grand Rapids. A comparison of the sequences of the clones obtained from WT and the Tnt6a mutants indicated that *Tnt1* is inserted into the intron of the *LsGA3ox1* gene (Fig. 1B). Thus, *Tnt1* may have been removed with the intron during splicing. To examine this possibility, we performed expression analysis by quantitative reverse transcription (QRT)-PCR.

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Abbreviations: DMSO, dimethyl sulfoxide; GA, gibberellin; hnRNA, heterogeneous nuclear RNA; ORF, open reading frame; QRT-PCR, quantitative reverse transcription-polymerase chain reaction; WT, wild type

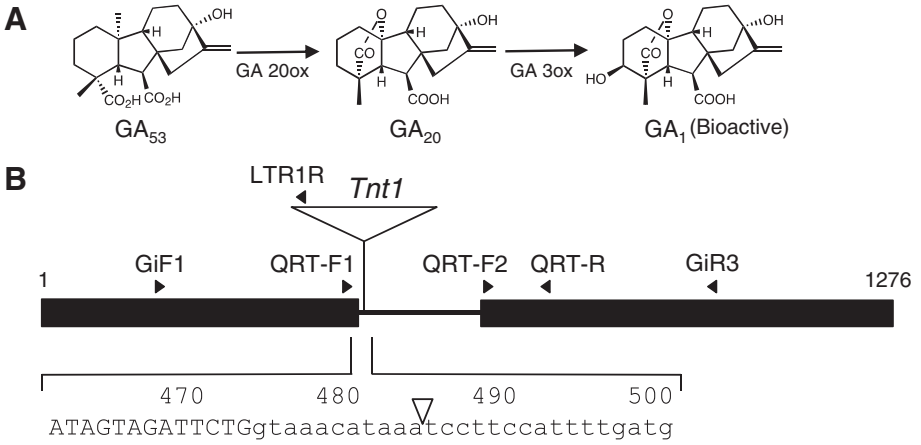

Fig. 1. A Mutation in the *LsGA3ox1* Gene of the Tnt6a Lettuce Mutants.
A, Conversion steps carried out by gibberellin 20-oxidase (GA 20ox) and GA 3-oxidase (GA 3ox). B, Diagram of the *LsGA3ox1* gene. Black boxes, exons in ORF; inverted open triangles, *Tnt1* insertion in Tnt6a mutant; filled triangles, primers used in this study. The region from 461 to 500 is closed up.

The QRT-PCR procedure, which utilized SYBR Green I and Thermal Cycler Dice Real-Time System TP800 (Takara, Shiga, Japan), was the same as described previously.[5] The transcript levels of *LsGA3ox1* in 15-d-old seedlings (Fig. 2B) are shown in Fig. 3A. Primer sets QRT-F1 (5′-TGGCCCAACCGTTATAGTAG-3′, sense)-QRT-R (5′-CTTGTGATCCATCCCATTTTACG-3′, antisense) and QRT-F2 (5′-TGATGTAATCGAAGGGTATAAACAC-3′, sense)-QRT-R (Fig. 1B) were used to detect mature *LsGA3ox1* mRNA and total *LsGA3ox1* transcripts, respectively. The total transcripts included mature mRNA and unspliced heterogeneous nuclear RNA (hnRNA). The phenotype of the Tnt6a mutants at the early growth stage was not significantly different from that of WT Jessy (Fig. 2B). Nevertheless, the levels of mature *LsGA3ox1* mRNA in the Tnt6a mutants were much lower (approximately 1/1,000) than those found in WT, while the levels of total transcripts in the Tnt6a mutants were approximately 1/8 of the WT levels (Fig. 3A). These results suggest that the levels of total *LsGA3ox1* transcripts in the Tnt6a mutants reflected the hnRNA levels, and consequently that the *LsGA3ox1* gene was nearly knocked out in the Tnt6a mutant seedlings.

Next, we quantified the transcripts of the GA biosynthetic genes in the stems of 50-d-old lettuce plants (Fig. 2C–E). The nucleotide sequences of the primer sets for *LsGA3ox2*, *LsGA20ox1*, and *LsGA20ox2* were reported in our previous study.[5] The stems of 50-d-old Tnt6a mutant plants were shorter than those of the WT. The levels of mature *LsGA3ox1* mRNA in the 50-d-old Tnt6a mutant plants were substantially lower than the WT (Fig. 3B). The transcript levels of *LsGA20ox1* and *LsGA20ox2* in the Tnt6a mutants were higher than in the WT, while there were no significant differences in *LsGA3ox2* expression levels between in the Tnt6a mutants and the WT (Fig. 3B). This suggests that *LsGA20ox1* and *LsGA20ox2*, but not *LsGA3ox2*, operate under positive feedback regulation in the stems. The transcript levels of *GA 20-* and *GA 3-oxidase*s are upregulated in GA-deficient mutants by feedback regulation in other plant species,[1] but not all GA biosynthetic genes are regulated by feedback mechanisms. For example, the mRNA levels of *AtGA3ox1*

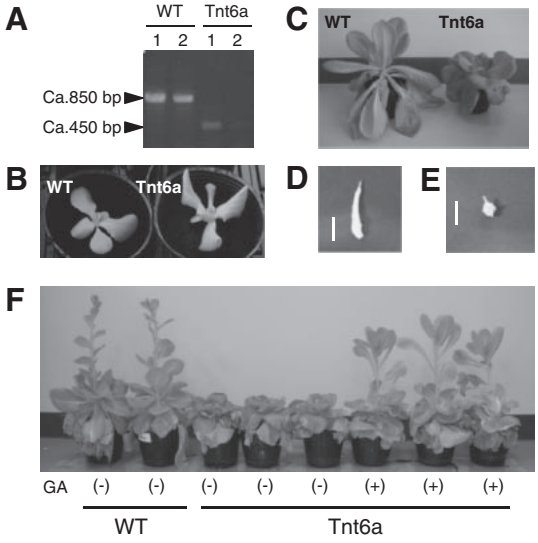

Fig. 2. Phenotype of the Tnt6a Lettuce Mutants.
A, PCR genotyping of wild-type (WT) lettuce plants and homozygous Tnt6a mutants (Tnt6a). B, Fifteen-d-old lettuce seedlings. C, Fifty-d-old lettuce plants. D, Stem from 50-d-old lettuce WT plant. White bar, 1 cm. E, Stem from 50-d-old lettuce Tnt6a mutant. White bar, 1 cm. F, Complementation of the dwarf phenotype of Tnt6a by application of bioactive GA. The photograph shows the plants 30 d after treatment. (−), sprayed with 0.1% DMSO solution without GA_3; (+), sprayed with 0.1% DMSO solution with GA_3.

(*GA4*), but not of *AtGA3ox2* (*GA4H*), are upregulated in the seeds of *Arabidopsis ga1-3*, a GA-deficient mutant.[11] Tnt6a mutants germinate and grow normally until the early growth stage,[9] perhaps due to functional redundancy provided by *LsGA3ox2*, but the Tnt6a mutants exhibited much shorter stems at the bolting stage as compared with the WT, suggesting that *LsGA3ox1* is critical to stem elongation. A previous report also suggested a critical role for *LsGA3ox1* in stem elongation.[6] *GA4* and *GA4H* are functionally redundant in seed germination and stem elongation in *Arabidopsis*.[12] Thus, the regulation patterns and roles of the GA 3-oxidase gene paralogs depend on the plant species.

In the present study, although the expression of *LsGA20ox*s was upregulated in the stems, the 50-d-old

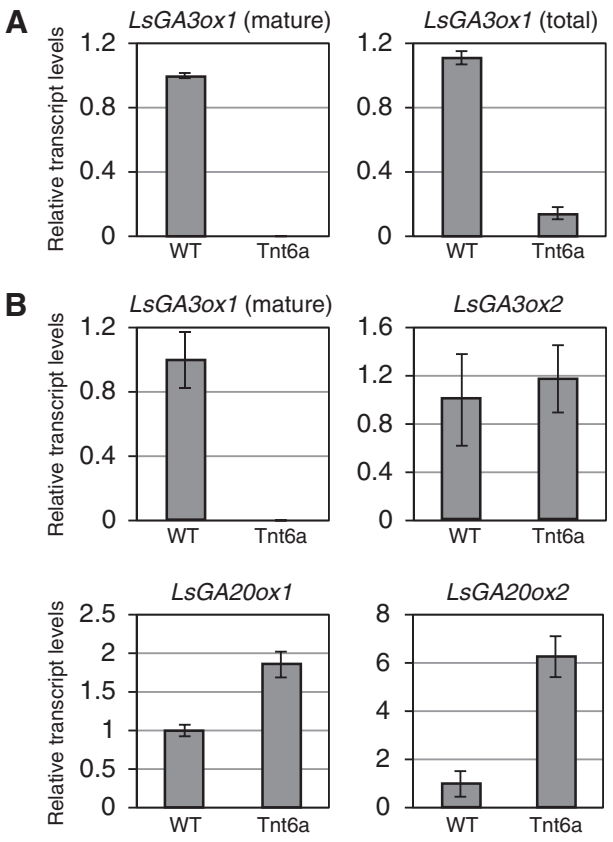

Fig. 3. Expression Analysis of Gibberellin Biosynthetic Genes in Tnt6a Mutants.

Expression analyses were performed by QRT-PCR. The results were normalized to the expression of 18S rRNA (internal control). A, Transcript levels of *LsGA3ox1* in the 15-d-old seedlings. The left graph shows the levels of *LsGA3ox1* mature mRNA. The right graph shows the levels of total *LsGA3ox1* transcripts, including mature mRNA and unspliced hnRNA. The expression levels of the samples examined are given relative to the reference value of the level of *LsGA3ox1* mature mRNA in WT, set to 1. The bars show the standard error (WT, n = 2; Tnt6a, n = 3). B, Transcript levels of *LsGA3ox*s and *LsGA20ox*s in the stems of the 50-d-old plants. The expression levels of the samples examined are given relative to the reference value of the level of each transcript in WT, set to 1. Bars show standard error (WT, n = 2; Tnt6a, n = 6).

Tnt6a mutant plants exhibited a dwarf phenotype with stems shorter than WT (Fig. 2D and E). We applied bioactive GA to 50-d-old Tnt6a mutant rosette plants by spraying them with 5 mL of 10^{-5} M GA_3 solution. As solvent control, 0.1% dimethyl sulfoxide (DMSO) solution was applied by spraying. The phenotype of the Tnt6a mutants 30 d after GA treatment is shown in Fig. 2F. The average length of the stems of the GA-treated Tnt6a mutants was much longer than control Tnt6a, and was almost the same as the WT, although the shape and size of the cauline leaves of the mutant Tnt6a plants were different from the WT. Cauline leaves of Tnt6a mutants might expand due to application of GA_3. Taken together, we conclude that the shorter stems found in the Tnt6a mutants resulted from GA deficiency caused by a loss of *LsGA3ox1* function. To confirm our conclusion, the endogenous levels of GAs in the mutants should be quantified.

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