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Cloning and Characterization of Δ^1-Pyrroline-5-Carboxylate Synthetase Genes and Identification of Point Mutants in *Medicago truncatula*

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ABSTRACT To tolerate environmentally adverse conditions such as cold, drought, and salinity, plants often synthesize and accumulate proline in cells as compatible osmolytes. Δ^1-Pyrroline-5-carboxylate synthetase (*P5CS*) catalyzes the rate-limiting step of proline biosynthesis from glutamate. Two complete genes, *MtP5CS1* and *MtP5CS2*, were isolated from the model legume *Medicago truncatula* by cDNA cloning and bacterial artificial chromosome library screening. Nucleotide sequence analysis showed that both genes consisted of 20 exons and 19 introns. Alignment of the predicted amino acid sequences revealed high similarities with P5CS proteins from other plant species. The two *MtP5CS* genes were expressed in response to high salt and low temperature treatments. Semi-quantitative reverse transcription-polymerase chain reaction showed that *MtP5CS1* was expressed earlier than *MtP5CS2*, indicating differential regulation of the two genes. To evaluate the reverse genetic effects of nucleotide changes on *MtP5CS* function, a Targeting Induced Local Lesions in Genomes approach was taken. Three mutants each were isolated for *MtP5CS1* and *MtP5CS2*, of which a *P5CS2* nonsense mutant carrying a codon change from arginine to stop was expected to bring translation to premature termination. These provide a valuable genetic resource with which to determine the function of the *P5CS* genes in environmental stress responses of legume crops.

Keywords : *medicago truncatula*, model legume, stress response, proline, Δ^1-pyrroline-5-carboxylate synthetase, nonsense mutant

Medicago truncatula is a model legume that has extensively been used for genomics research for the past decade. A small diploid genome (2n = 16; 450 Mbp/1C), a self-fertilizing reproductive habit, and the capacity for transformation and regeneration are some of the attributes that made this Mediterranean-originated species amenable to structural and functional genomic analyses (Barker *et al.*, 1990; Blondon *et al.*, 1994; Trieu *et al.*, 2000). A large collection of valuable resources have accumulated in *M. truncatula* that are accessible for studies to resolve physiological processes unique to legumes (VandenBosch and Stacey, 2003). In addition to the hallmark ability to establish symbiosis with nitrogen-fixing soil bacteria and phosphorus-supplying endomycorrhiza, legumes have been useful for studying plant responses to pathogenic and environmental stresses. *M. truncatula* is particularly well adapted to semi-arid conditions and winter-growing fields, encouraging its use for studies elucidating signaling pathways in response to drought, high salinity, and low temperature.

Proline is one of the important osmolytes that help plants to counter dehydration by increasing cellular osmotic potential. Proline is synthesized from both glutamate and ornithine (Buchanan *et al.*, 2000). The glutamate pathway, considered as the major biosynthetic route, is comprised of two steps. In the first step, glutamate is phosphorylated to γ-glutamyl phosphate and subsequently reduced to glutamic-γ-semialdehyde (GSA) by a bifunctional enzyme Δ^1-pyrroline-5-carboxylate synthetase (P5CS). The second step involves spontaneous cyclization of glutamic-γ-semialdehyde into Δ^1-pyrroline-5-carboxylate (P5C) and subsequent reduction to proline by pyrroline-5-carboxylate reductase (P5CR). P5CS, a rate-limiting enzyme, serves as an important determinant of the cellular proline level. In many plants, the expression of *P5CS* genes is induced by osmotic stress (Yoshiba *et al.*, 1995; Igarashi *et al.*, 1997; Ginzberg *et al.*, 1998). Two *P5CS* genes (*MtP5CS1* and *MtP5CS2*) were isolated from *M. truncatula*, of which only one (*MtP5CS1*) was

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extensively characterized with a full-length cDNA clone (Armengaud *et al.*, 2004). Despite much debated osmoprotective roles of proline in the dehydrated cells (Seki *et al.*, 2003), little is known about how the biosynthetic and degradative pathways are coordinated to adjust its level. Thus, it is of interest to address what physiological consequences would result if any of the proline biosynthetic or degradative genes is knocked out.

Reverse genetic approaches recently emerged as a potent tool to investigate gene function once the nucleotide sequence of the target gene is known. They include diverse technologies such as T-DNA or transposon tagging (Bouché and Bouchez, 2001), RNA interference (Baulcombe, 2004), and TILLING (Targeting Induced Local Lesions in Genomes) (McCallum *et al.*, 2000). TILLING utilizes DNA populations derived from mutagenized M_2 plants that carry point mutations. DNA samples isolated from each plant are pooled and screened with primers specific to a gene of interest whose sequence has been determined. Mutants are searched for by electrophoretically tracing cleavage fragments from heteroduplexes that are derived from melting and reannealing of pooled DNA samples. A mutant with a nucleotide change in the translatable region of the gene would then provide a material for analyzing phenotypic effects of the impaired gene function (Comai and Henikoff, 2006).

Here, two complete *P5CS* genes were isolated from the model legume *M. truncatula*. Their expression patterns were characterized in response to abiotic stress treatments and effects on the cellular proline levels were examined. The genomic and full-length cDNA sequences of *MtP5CS* genes facilitated TILLING, which identified several point mutants including one with a predictable genetic effect.

MATERIALS AND METHODS

Plant material and stress treatments

Seeds of *M. truncatula* genotype A17 were germinated in the absence of light at 4℃ for 48 h. Seedlings were placed between two layers of Whatman paper pre-wet with the Murashige Skoog (MS) medium (Duchefa Biochemie) in a square plastic dish (12.5×12.5×2 cm^3) and grown at a semi-vertical position at 25℃ in a growth chamber with a daily cycle of 14 h of light and 10 h of dark for 10 d. For salinity treatments, NaCl was added to the MS medium at the final concentration of 200 mM. Seedlings were also placed at 4℃ or at 50% relative humidity for low temperature (cold) treatments. Seedlings collected at several time points were frozen in liquid nitrogen and stored at -80℃.

BAC library screening

The *Hin*dIII and *Bam*HI bacterial artificial chromosome (BAC) libraries of *M. truncatula* (Nam *et al.*, 1999; Park and Nam, 2006) were screened with cDNA fragments amplified from cold-treated *M. truncatula* RNA using specific primers for *MtP5CS1* (forward: 5'-aggctaatcgatcaaatgcc-3', reverse: 5'-atctactacttgtccattacc-3') and for *MtP5CS2* (forward: 5'-ggtgttcaactatatggtgg-3', reverse: 5'-caagacattatgcgcatacc-3'). Filter hybridization screening and confirmative PCR screening of BAC DNA multiplex pools were carried out as described (Song and Nam, 2005). Plasmid DNA was isolated from positively identified BAC clones and analyzed by *Not*I digestion and pulsed field gel electrophoresis (PFGE) as described (Park and Nam, 2006). Analysis of nucleotide sequences and multiple alignment were carried out using the Clustal W method (Thompson *et al.*, 1994) and the BioEdit program.

cDNA cloning

Total RNA was isolated from NaCl-treated shoots using the TRIZOL reagent (Molecular Research). First-strand cDNA was synthesized from 1 μg of total RNA using MMLV Reverse Transcriptase (Ambion) following the manufacturer's protocol and subsequently cloned into Gateway cloning vectors (Invitrogen). To clone a full-length *MtP5CS2* cDNA, PCR was carried out using primers that contained the start and stop codons and built-in overhangs (12attB1_p5cs2_fc: 5'-aaaaagcaggcttgctccatggatccaacacgag-3', 12attB2_p5cs2_fc: 5'-agaaagctgggtgccattatgcttttacttgcag-3'). The 2.2-kb fragment, amplified using Phusion high-fidelity DNA polymerase (FINNZYMES), was purified from the gel, concentrated, and subjected to recombination with the pDONOR221 vector in the presence of BP clonase II.

Genetic mapping

Strategies and PCR conditions for intron-targeted mole-

cular markers were described by Choi *et al.* (2004). For *MtP5CS1* genetic marker, PCR primers were re-designed to contain *Nsi*I site (forward: 5'-cttatttaaatttgaaaacgtctacaatgc-3' and reverse: 5'-gttgtcaaattatcatttaattgg-3') to reveal single nucleotide polymorphism by means of the dCAPS method (Neff *et al.*, 1998). For purposes of genotyping, F_2 mapping population produced from a cross between *M. truncatula* ecotypes A17 and A20 was used (Choi *et al.*, 2004). To map *MtP5CS2* gene, genomic information from BAC-by-BAC whole genome sequencing (http://medicago.org/genome/) was used and corresponding sequence position was anchored to the integrated *M. truncatula* genetic map.

Reverse transcription-polymerase chain reaction (RT-PCR)

First-strand cDNAs synthesized from *M. truncatula* seedlings collected at various time points were used as templates for PCR with specific primers for *MtP5CS1* (forward: 5'-aggctaatcgatcaaatgcc-3', reverse: 5'-atctactacttgtccattacc-3') and *MtP5CS2* (forward: 5'-gcaggaaatggtggactgaatgaac-3', reverse: 5'-gtataaatcatacttcgatcgccatc-3'). The details of PCR conditions were as described (Song and Nam, 2005). The *MtACTIN* and *MtCOR47-like* genes (Song and Nam, 2005) were used as internal control and stress-specific markers, respectively. To amplify the *MtPDH* gene, specific primers (forward: 5'-gttgatggccaccagagttatcc-3', reverse: 5'-cctttgagattgcgaatcacagc-3') were used.

Proline content measurement

Proline contents were measured using the acid ninhydrin method (Troll and Lindsley, 1995). Briefly, lyophilized seedlings were extracted with water, centrifuged, and the supernatant was mixed with equal volumes of glacial acetic acid and acid ninhydrin reagent. The mixture was subsequently heated at 100℃, cooled down, and partitioned against toluene, and the absorbance of the organic phase was measured at 515 nm. The resulting values were compared with a standard curve drawn using the known amounts of proline.

TILLING

Ethylmethanesulfonate (EMS)-mutagenized *M. truncatula* M_2 DNA populations (Penmetsa and Cook, 1997) were screened with fluorescently labeled oligonucleotide primer pairs derived from CODDLE analysis (http://www.proweb.org) as described by Perry *et al.* (2003). The pooled DNA stocks were screened by PCR amplification with the primer pairs for *P5CS1* (*P5CS1*-T3F: 5'-ttctggcatattttgggataatgacag-3' and *P5CS1*-T3R: 5'-tatgtgaactgtctcttgccttctc-3') and for *P5CS2* (*P5CS2*-T3F: 5'-aatggatcaatagatagctgcattgg-3' and *P5CS2*-T3R: 5'-aagatcacaatttaatactacgccgg-3') that target the conserved γ-glutamyl kinase domain (*P5CS1*) and NAD(P) binding site (*P5CS2*), respectively. The PCR-amplified fragments were subsequently denatured and reannealed to allow formation of heteroduplexes. Mismatch cleavage by *Cel*I (Till *et al.*, 2004) and electrophoretic analysis using denaturing polyacrylamide gels were carried out following published protocols (Till *et al.*, 2006). All the procedures were carried out in Dr. Douglas R. Cook's laboratory at the University of California, Davis, U. S. A.

RESULTS

Cloning and sequence analysis of *MtP5CS* genes

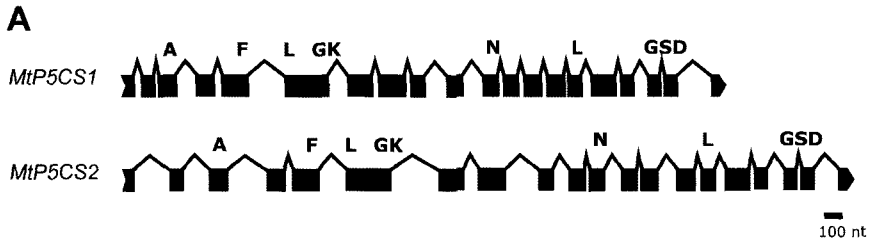
Filter hybridization screening of *Hin*dIII and *Bam*HI BAC libraries yielded three and eleven positive clones for *MtP5CS1* and *MtP5CS2* genes, respectively (Table 1). Using these BAC clones, nucleotide sequences were determined for the entire genomic regions of the two genes. From cDNA synthesized from salt-treated seedlings, full-length

Table 1. BAC clones identified with *MtP5CS1* and *MtP5CS2* gene probes

Probe[†]	No. of hits	BAC clones hybridized (insert size in kb)[‡]
MtP5CS1	3	H34J18(95), H60H18(80), H61L17(55)
MtP5CS2	11	B05N14(95), B08H04(100), B13K03(160), B21C02(100), B28A06(90), B31H16(100), B37B21(105), B40K10(105), B42A23(125), B47K03(125), B58O16(95)

[†]*MtP5CS1* and *MtP5CS2* probes were used to screen *Hin*dIII (H) and *Bam*HI (B) BAC libraries, respectively.
[‡]Positive BAC DNAs were isolated and analyzed by *Not*I digestion and PFGE.

P5CS1 and *P5CS2* clones were also isolated. Comparison between the genomic and cDNA sequences revealed that both *MtP5CS* genes consisted of 20 exons and 19 introns (Fig. 1A). However, *P5CS2* (7,713 nucleotides; Fig. 1B) was considerably longer than *P5CS1* (6,003 nucleotides; not shown) due to differences in intron size. Eighteen



B

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ATGGATCCAACACGAGCTTTTGTCAAGAGTGTTAAACGTGTTGTTGTTAAGgtgaatatataacaatttatattacaacgttgctttctcttctgttttt 100
 M  D  P  T  R  A  F  V  K  S  V  K  R  V  V  V  K                                                  (17)
ttgattgatctaataataataataacgttgtttagtgtttttacgtggttgaaaaaaatactattgagatcgatgtggtatatggattgatatgaatg 200
ttgtcaatgtgatttcttttgttctttcgatatttttatttattagtggctatttttttataatatatttgttacatgtatgatataggatgatgagatc 300
tcgacatgattgttaattttgatgtgttttgagttatcaaattggttttcttatgttatgtattttatcgatgatattgtttatttgtaaaaatattaag 400
tgttaagtatactgagctatcaaaatggttttttttgtgtactattggattttatttatttatttatttatttatttttgactttgtatgaatttgtaactc 500
atcgatggtatcattgcatttatttttttaataatcatgtgatggttgtataattaactatttggttcttttgtgatatatgaagGTTGGAACAGCTGTG 600
                                                                                    V  G  T  A  V  (22)
GTTACAAGAAGTGATGGAAGATTAGCATTGGGAAGACTTGGAGCTCTTTGTGAACAGgtgatgatttatagcattttacactattaaccactgtgtcaag 700
 V  T  R  S  D  G  R  L  A  L  G  R  L  G  A  L  C  E  Q                                          (41)
ggttcaaaccctcgaaactaacataactcgtgaagttattaaaataaaatcatttacttatgagaaaatatgtaactagtaataacggaaccagtgaaac 800
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aattcttcattaatcatgtgattattttaactggaagctatttttttctgcagCTTAAAGAGCTAAATACAAGAGGATATGAAGTCATACTGGTGACTTCA 1100
                                                    L  K  E  L  N  T  R  G  Y  E  V  I  L  V  T  S  (57)
GGAGCAGTTGGTCTTGGCAGGCAAAGACTAAGATATCGCCGATTGGCCAATAGCAGgttttttttgtaatctatctttttgctagagcaactatacatttttc 1200
 G  A  V  G  L  G  R  Q  R  L  R  Y  R  R  L  A  N  S  S                                          (76)
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                                                F  S  D  L  Q  N  P  Q  Y  E  L  D  G  K  A  C  A  A  (94)
TTGGGCAGAGCAGTCTCATGGCTCTCTATGATATCATGTTTAGTCAGgtaaacataatattaagcttctaattcaatatgtcgcgaatcttgtatatgtg 1900
V  G  Q  S  S  L  M  A  L  Y  D  I  M  F  S  Q                                                    (110)
tgtatttctttgacatatgaactatttttctattaatcgaactgattccttcttattgatgtgattttccgtgatgcagCTTGATGTGACTTCATCACAA 2000
                                                                                 L  D  V  T  S  S  Q  (117)
CTTCTTGTGAATGATGGATTTTTTCGGGATGCAGATTTCAGAAAACAACTAACTGACACTGTGCACTCGTTATTAGAGTTAAGGGTCATCCCCATTTTCA 2100
 L  L  V  N  D  G  F  F  R  D  A  D  F  R  K  Q  L  T  D  T  V  H  S  L  L  E  L  R  V  I  P  I  F  (150)
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N  E  N  D  A  V  S  T  R  K  A  P  Y  E                                                           (164)
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                                                L  L  Q  D  S  S  G  I  F  W  D  N  D  S  L  A  G  L  L  (183)
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TATTCAGGCACACCTGTGATTATCACCAGgtatcatgtagtgtttaaagtagactattgacactgaaaatcactatttttttgtttcgattttcgatacaa 2900
 Y  S  G  T  P  V  I  I  T  S                                                                      (260)
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Fig. 1. Continue

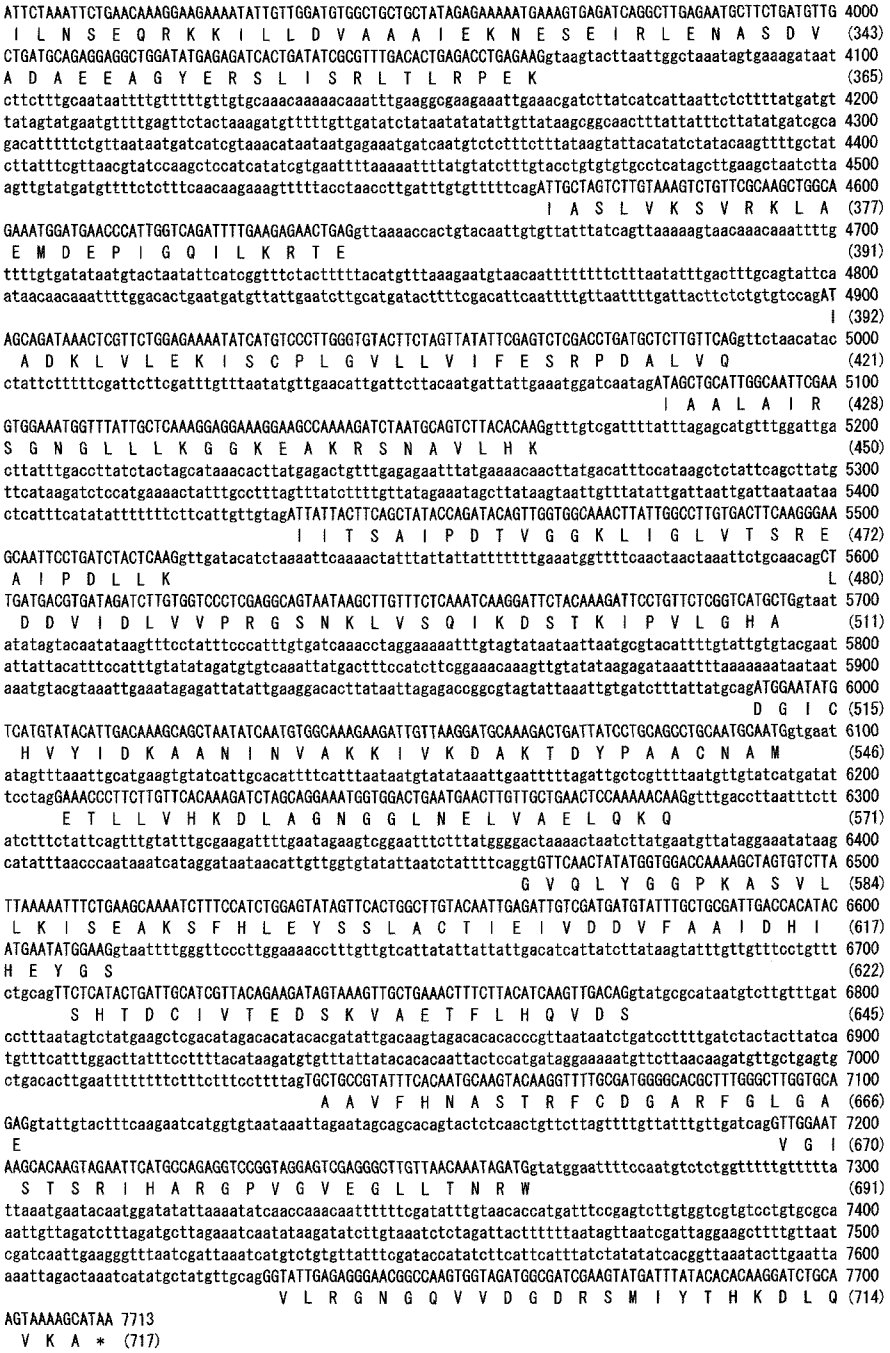

Fig. 1. Gene structure of *MtP5CS1* and *MtP5CS2*. **A.** Schematic representation of the exon-intron arrangements. Exons are represented by black boxes and introns by dented black lines. Letters above the boxes designate the functional domains of the predicted polypeptides encoded by the two genes. A: ATP-binding site, F: feedback inhibition site, GK: conserved γ-glutamyl kinase domain, GSD: conserved glutamic-γ-semialdehyde dehydrogenase domain, L: putative leucine domain, Lz: conserved leucine zipper, and N: NAD(P)H-binding site. **B.** Complete genomic sequence of *MtP5CS2*. Numbers designate nucleotide (amino acid in parentheses) positions starting from the predicted translation initiation site.

(*P5CS1*) (not shown) and all nineteen (*P5CS2*) introns (Fig. 1B) of the two genes, respectively, started with GT and ended with AG, a feature widely conserved in many *M. truncatula* genes (Zhu *et al.*, 2003; Choi *et al.*, 2004).

The predicted amino acid sequences of *MtP5CS* genes were compared with each other and with the homologous sequences from other species (Fig. 2). Alignment showed the overall sequence similarity of 73.7% between the two MtP5CS proteins. Strong similarity was also evident between MtP5CS and other plant P5CS proteins (72.8-90.2%). By contrast, much weaker yet significant similarities were observed with *E. coli* γ-glutamyl kinase (27%) and glutamic-γ-semialdehyde dehydrogenase (GSA-DH) (35%), the two separate enzymes corresponding to the N-terminal and C-terminal halves of P5CS, respectively. In addition to these two core supradomains, several conserved structural motifs were also identified in MtP5CS proteins, including an ATP-binding site, a leucine zipper domain, and a putative NAD(P)H-binding site (Fig. 1A and Fig. 2).

Genetic mapping of *MtP5CS* genes

Genotyping with F_2 *M. truncatula* mapping population (Fig. 3A) and calculation of genetic distance revealed that *P5CS1* marker positioned at 56.1 cM of linkage group (LG)-3, where the gene was flanked by GSb and DK201R markers (Fig. 3B). This genetic position was reconfirmed by a combination of BlastN and Blast2 homology analyses. As a result, a BAC clone, CT963134, was identified to

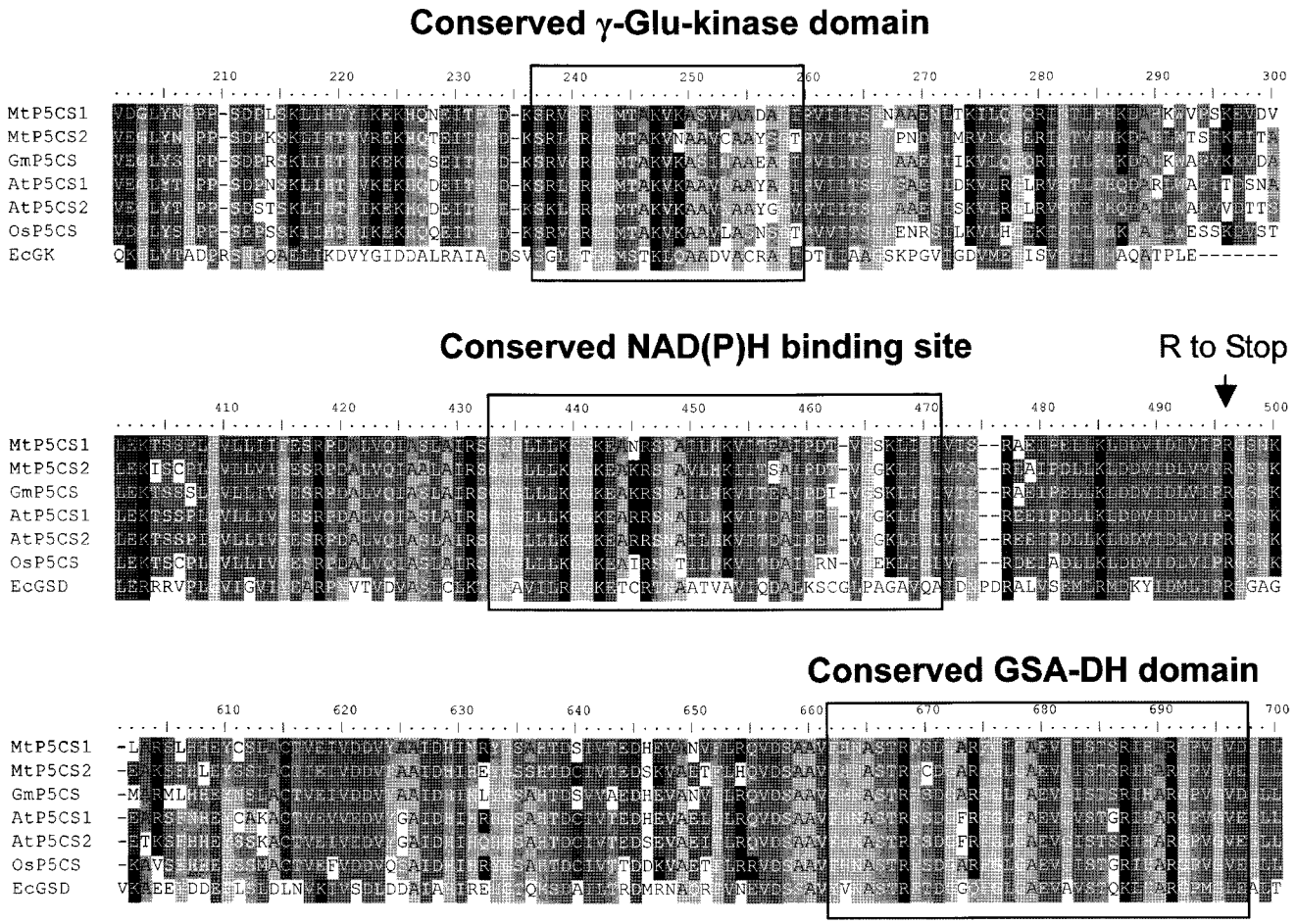

Fig. 2. Comparison of the predicted amino acid sequences of *MtP5CS1* and *MtP5CS2* with proteins from other species. Four plant P5CS and *E. coli* γ-glutamyl kinase (EcGK) and glutamic-γ-semialdehyde dehydrogenase (EcGSD) sequences are aligned and part of the regions around three representative functional domains are shown. Genbank accession numbers are: AtP5CS1 (P54887; *Arabidopsis thaliana*), AtP5CS2 (P54888; *A. thaliana*), EcGK (Q0TL74; *Escherichia coli*), EcGSD (Q0TL73; *E. coli*), GmP5CS (AAR86688; *Glycine max*), MtP5CS1 (AJ278818; *M. truncatula*), and OsP5CS (BAA19916; *Oryza sativa*). A point mutation identified by TILLING (see text) that converted an arginine (R) to a stop codon is shown.

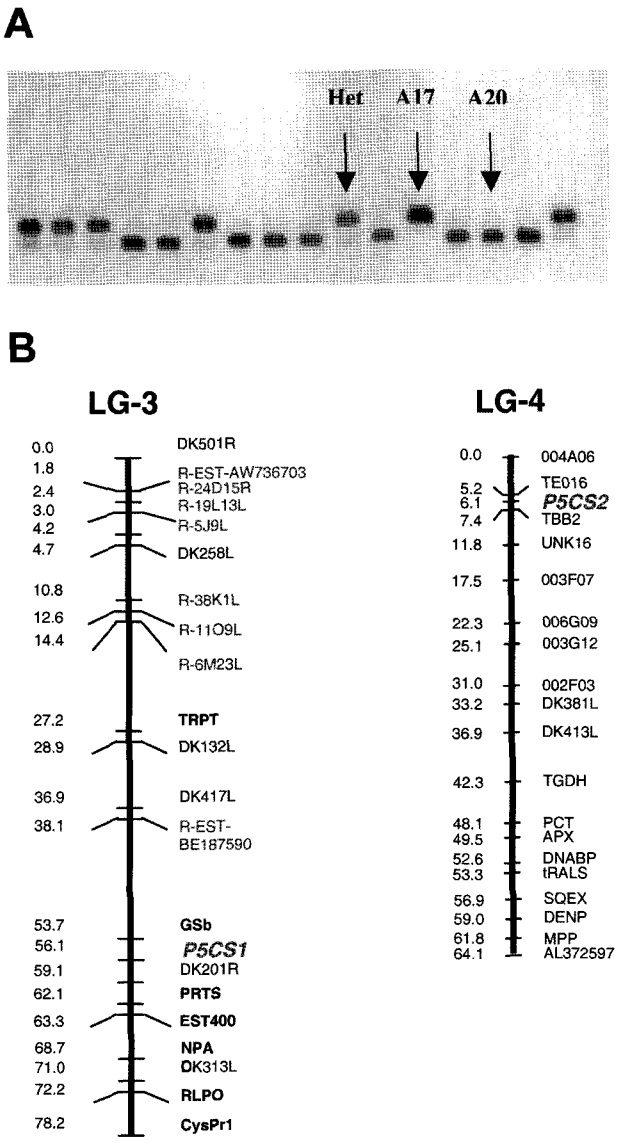

Fig. 3. Genetic mapping of *MtP5CS1* and *MtP5CS2*. **A.** An example of F_2 screening by PCR using *MtP5CS1*-specific dCAPS primers. Arrows indicate DNA fragments amplified from A17-type, A20-type, or heterozygous (Het) individuals. **B.** Map positions of *MtP5CS1* and *MtP5CS2* relative to the molecular markers of *M. truncatula*.

contain the corresponding *P5CS1* genomic sequence with complete identity and consistent genetic position.

In the case of *P5CS2*, map position was searched for by analyses at the *M. truncatula* whole genome sequencing web site (http://medicago.org/genome/). BlastN analysis with *P5CS2* coding sequence identified a BAC clone, AC148916, whose genomic sequence was located in LG-4. More detailed analysis for physical mapping data revealed that the target BAC clone formed a contig consisting of 62 BAC clones, of which three BAC clones (AC148916, AC174285, and AC144658) were in the genome sequencing pipeline. Of these three BACs, only one BAC clone, AC144658, was genetically mapped at 6.1 cM of MtLG-4. Contig relationship of target BAC with AC144658 was re-ensured by aligning BAC end sequences, thereby confirming that *P5CS2* mapped to the same genomic site with AC144658. This genetic marker is located close to other gene-based markers, such as TE016 and TBB2 markers (Fig. 3B).

Expression of *MtP5CS* genes in response to stress treatments

To examine the expression patterns of *MtP5CS* genes under environmental stress, seedlings exposed to high salt and low temperature (cold) conditions were used for RNA isolation. Semi-quantitative RT-PCR revealed that the expression of *MtP5CS* genes was up-regulated by both treatments. Nevertheless, temporal changes in the accumulation of *P5CS1* and *P5CS2* transcripts varied significantly (Fig. 4A). Whereas *P5CS1* transcripts were first detected at 6 h, reached a maximal level at 12 h, and declined thereafter after NaCl treatment, *P5CS2* transcripts were only detected considerably at 12 h and increased steadily throughout the remaining periods after the same treatment. In cold-treated seedlings, *P5CS1* transcripts began to accumulate early as well and reached maximal levels between 12-48 h, whereas *P5CS2* transcripts accumulated much slowly, reaching a substantial level only at 72 h after treatment. These observations indicate that *MtP5CS1* was induced prior to *MtP5CS2* and that only *MtP5CS2* transcripts remained stable for an extended time period. Therefore, the *P5CS1* and *P5CS2* genes are likely regulated differentially in *M. truncatula*. The expression patterns of the *MtPDH* gene encoding proline dehydrogenase (PDH) appeared to be inversely correlated with the expression of *MtP5CS1* to a certain extent, as its transcripts accumulated when *MtP5CS1* expression declined at 24 h in response to NaCl treatment and at 48 h in response to cold treatment.

Correlation between *MtP5CS* gene expression and proline levels

The effects of changes in *MtP5CS* gene expression on the cellular proline levels in salt- and cold-treated *M. truncatula* seedlings were evaluated by proline content measurement (Fig. 4B). After either treatment, the levels of proline increased gradually, reached maximal levels at 24 h, and then decreased from 48 h. The reduced proline levels remained as such throughout the rest of the periods, although the extent of decrease was more pronounced in cold-treated than in salt-treated seedlings. The reduction of proline levels appeared to be coincident with the emergence of *MtPDH* transcripts at 24-48 h, indicating a possible role of proline degradation in the regulation of the intracellular proline level.

Isolation of point mutants

To evaluate the functional effects of mutations in *MtP5CS1* and *MtP5CS2* loci at the whole plant level, TILLING was carried out. Oligonucleotide primers specific to the *MtP5CS1* and *MtP5CS2* genes that targeted the domains most likely to suffer devastating functional effects once a mutation would occur were designed and used to amplify PCR fragments. An example of such analysis by the CODDLE program (Fig. 5) demonstrates the regions most labile to functional disruption from the prospective amino acid changes. Using fluorescently labeled primers, a multiplex pool of DNA was screened to isolate M_2 individuals descended from EMS-mutagenized seeds. Three independent mutants were isolated to carry single nucleotide polymorphisms in the *MtP5CS1* gene. However, all of these mutations were located in the introns, and therefore, no functional effects were expected to occur (Table 2). Likewise, three independent mutants were isolated to have lesions in the *MtP5CS2* genes. Two of these mutations were located in the introns and one in an exon (Table 2). A prediction from this latter

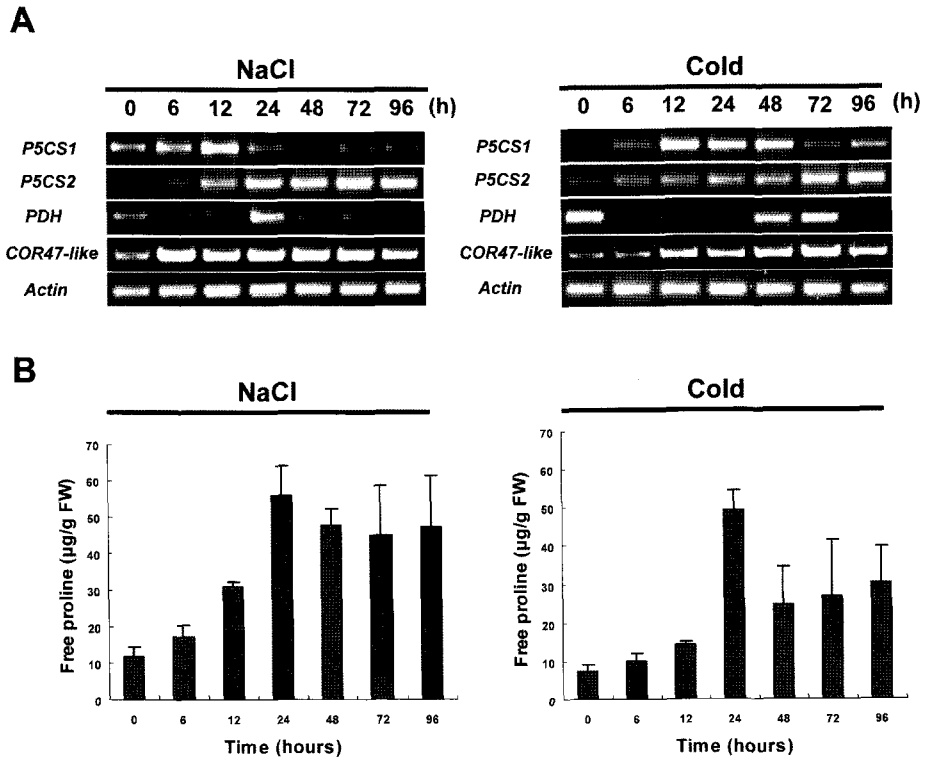

Fig. 4. Expression of proline metabolic pathway genes and proline contents in *M. truncatula* seedlings under stress conditions. **A.** RT-PCR analysis of *MtP5CS1*, *MtP5CS2*, and *MtPDH* in salt and low temperature (cold)-treated seedlings. Hours (h) after each treatment are shown. *MtCOR47-like* and *MtActin* are used as a stress-specific marker and an internal control, respectively. **B.** Proline contents measured at the corresponding time points to (A). Values are the means of three independent measurements. Bars represent standard errors.

Table 2. Results from the TILLING analysis

Gene	M_2 lineage†	Nucleotide position‡	Nucleotide change	Zygotic status	Genetic effect
MtP5CS1	19281	2080	G to A	Hetero	in intron
	10570	2140	C to T	Hetero	in intron
	11852	2267	C to T	Hetero	in intron
MtP5CS2	10523	5330	C to T	Homo	in intron
	11720B	5352	G to A	Hetero	in intron
	19005	5629	C to T	Homo	R to stop

†Serial number of the EMS-mutagenized M_2 individuals of *M. truncatula*
‡Starting from the translation initiation site (ATG), e.g. as shown in Fig. 1B

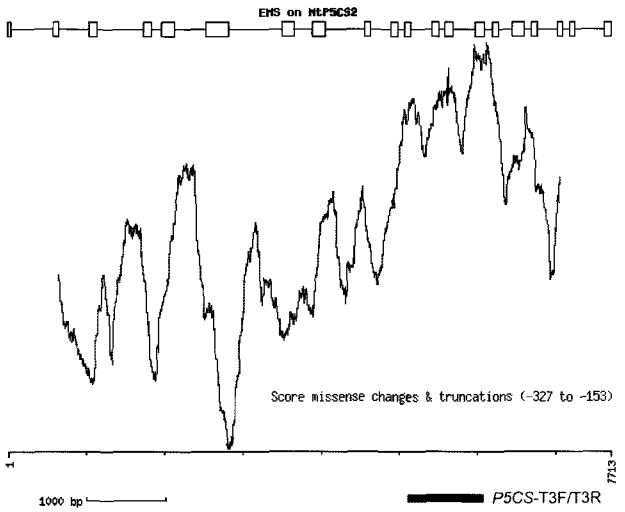

Fig. 5. An output of the CODDLE program showing the prospective effects of EMS mutagenesis on *MtP5CS2* gene. The genomic sequence of *MtP5CS2* including the entire coding region (7,713 nucleotides) is represented by white boxes (exons) and black lines (introns) (top) and a ruler (bottom). Mutations that lead to silent, missense, and nonsense changes score differently and the sum of the scores around a window of 500 nucleotides at a given amino acid residue is plotted (http://www.proweb.org/glossary.html). The range of the plotted values (-327 to -153) is shown. A thick black line labeled with *P5CS*-T3F/T3R (bottom) represents an amplicon covering the region within which *MtP5CS2* mutations were detected.

mutation indicated that a nonsense mutation would occur in the translated peptide sequences, altering arginine to a premature stop. Therefore, the termination of translation after the NAD(P)H-binding site of the P5CS2 protein (Fig. 2) would likely lead to the formation of a truncated polypeptide. Thus, this particular point mutant is likely to provide genetic material with which to examine the functional consequences of genetic mutation in *MtP5CS2* gene.

DISCUSSION

In this study genomic and cDNA clones were isolated for two *P5CS* genes from the model legume *M. truncatula*. Each of the two highly homologous *MtP5CS* genes encodes two connected functional supradomains that correspond to the bacterial γ-glutamyl kinase and glutamic-γ-semialdehyde dehydrogenase, respectively (Csonka and Hanson, 1991). Equipped with these dual enzymatic activities, P5CS presumably catalyzes the first step of proline biosynthesis from glutamate to P5C. Strong similarities were observed between *M. truncatula* and other plant P5CS proteins. A particularly high value (90.2%) of similarity was calculated with a soybean P5CS, indicating evolutionary conservation of the legume P5CS proteins. Consistently, a preliminary phylogenetic analysis showed distinction of the legume P5CS proteins from other taxonomic clades that contained species from different origins (not shown). The cDNA and BAC clones isolated in this study were useful for isolating point mutants by TILLING, for full genomic sequence with exact splice site information is often prerequisite to accurate primer design. Of these, *P5CS2* cDNA is the first reported full-length clone of the *P5CS2* gene in *M. truncatula*.

The complete genomic and cDNA clones of *MtP5CS* genes also facilitated their localization in *M. truncatula* genetic map. An intron-targeted dCAPS marker was deve-

loped for this purpose, which turned out to be an effective strategy to map such a sequence-characterized gene (Choi *et al.*, 2004). The two *P5CS* genes were genetically mapped in different linkage groups of *M. truncatula* genome, one (*P5CS1*) in LG-3 and the other (*P5CS2*) in LG-4. These data suggest a possible genomic event involved in *P5CS* gene evolution, for example translocation followed by tandem duplication. Despite the presence of two *P5CS* genes in *Arabidopsis thaliana* (Strizhov *et al.*, 1994), however, searches for genomic and expression profiling databases indicate that an additional member(s) is present in the *P5CS* gene family of *M. truncatula* (Kim and Nam, unpublished). This possibility, partly supported by the previous experiments in which three to four bands were recognized on a genomic Southern blot (Armengaud *et al.*, 2004), awaits further investigation.

The expression patterns of *MtP5CS1* and *MtP5CS2* in seedlings under salt and low temperature stresses were largely in accordance with the previous results that either gene was expressed highly yet in different manners in salt-treated seedlings (Armengaud *et al.*, 2004). Such differential expression patterns are commonly observed in many small gene families of plants (Ginzberg *et al.*, 1998; Strizhov *et al.*, 1997; Song and Nam, 2005). Here, we focused on the time course of transcript abundance in association with the changes in cellular proline level. As previously, the expression of *MtP5CS1* preceded that of *MtP5CS2*, the latter showing perseverance of the steady-state mRNA. However, cellular proline levels exhibited a sudden decrease at 48 h after treatments and remained as such thereafter. This observation, slightly different from the previous, appears to reflect the effects of the expression of *MtPDH* that encodes an enzyme catalyzing degradation of proline (Kiyosue *et al.*, 1996). Possibly, accumulation of proline that results from the elevated expression of *P5CS* genes gives a signal to activate its own degradative mechanism to protect cellular machinery from the toxicity of proline (Deuschle *et al.*, 2001). Thus, osmotically challenged plant cells seem to operate a monitoring system with which to control the intracellular proline concentration so that it does not exceed a certain threshold level.

Recent advances in genomic studies have enabled the elucidation of gene function once its nucleotide sequence is determined. TILLING particularly fits such reverse genetic approaches, as it identifies an individual with a point mutation. Using full genomic and cDNA sequence information, we attempted to analyze functions of the *MtP5CS* genes using mutants in which nucleotide sequences were altered. Indeed, TILLING identified a nonsense mutant of *MtP5CS2* in which translation of the polypeptide would be prematurely terminated. Preliminary examination of the M_3 seed isolates indicates that a significant proportion of the mutant seeds are unable to grow into seedlings, raising a possibility that *P5CS2* may encode proteins that are required for embryogenesis and early growth of *M. truncatula*. Thus, these seeds provide an opportunity to evaluate the physiological effects of gene knock-out on the accumulation of proline and stress tolerance of legume crops.

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REFERENCES

Armengaud, P., L. Thiery, N. Buhot, G. Grenier-De March, and A. Savoure. 2004. Transcriptional regulation of proline biosynthesis in *Medicago truncatula* reveals developmental and environmental specific features. Physiol. Plant. 120 : 442-450.

Barker, D. G., S. Bianchi, F. Blondon, Y. Dattee, G. Duc, S. Essad, P. Flament, P. Gallusci, G. Genier, P. Guy, X. Muel, J. Tourneur, J. Denarie, and T. Huguet. 1990. *Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium*-legume symbiosis. Plant Mol. Biol. Rep. 8 : 40-49.

Baulcombe, D. 2004. RNA silencing in plants. Nature 431 : 356-363.

Blondon, F., D. Marie, S. Brown, and A. Kondorosi. 1994. Genome size and base composition in *Medicago sativa* and *Medicago truncatula* species. Genome 37 : 264-270.

Bouché, N. and D. Bouchez. 2001. *Arabidopsis* gene knockout: phenotypes wanted. Curr. Opin. Plant Biol. 4 : 111-117.

Buchannan, B. B., W. Gruissem, and R. L. Jones. 2000. Bio-

chemistry and Molecular Biology of Plants. American Society of Plant Biologists, pp. 406-409.

Choi, H. K., D. Kim, T. Uhm, E. Limpens, H. Lim, P. Kalo, R. V. Penmetsa, A. Seres, O. Kulikova, T. Bisseling, G. B. Kiss, and D. R. Cook. 2004. A sequence-based genetic map of *Medicago truncatula* and comparison of marker colinearity with *Medicago sativa*. Genetics 166 : 1463-1502.

Comai, L. and S. Henikoff. 2006. TILLING: practical single-nucleotide mutation discovery. Plant J. 45 : 684-694.

Csonka, L. N. and A. D. Hanson. 1991. Prokaryotic osmoregulation: genetics and physiology. Annu. Rev. Microbiol. 45 : 569-606.

Deuschle, K., D. Funck, H. Hellmann, K. Daschner, S. Binder, W. B. Frommer. 2001. A nuclear gene encoding mitochondrial delta-pyrroline-5-carboxylate dehydrogenase and its potential role in protection from proline toxicity. Plant J. 27 : 345-356.

Ginzberg, I., H. Stein, Y. Kapulnik, L. Szabados, N. Strizhov, J. Schell, C. Koncz, and A. Zilberstein. 1998. Isolation and characterization of two different cDNAs of Δ^1-pyrroline-5-carboxylate synthase in alfalfa transcriptionally induced upon salt stress. Plant Mol. Biol. 38 : 755-764.

Igarashi, Y., Y. Yoshiba, Y. Sanada, K. Yamaguchi-Shinozaki, K. Wada, and K. Shinozaki. 1997. Characterization of the gene for Δ^1-pyrroline-5-carboxylate synthetase and correlation between the expression of the gene and salt tolerance in *Oryza sativa* L. Plant Mol. Biol. 33 : 857-865.

Kiyosue, T., Y. Yoshiba, K. Yamaguchi-Shinozaki, and K. Shinozaki. 1996. A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in *Arabidopsis*. Plant Cell 8 : 1323-1335.

McCallum, C. M., L. Comai, E. A. Greene, and S. Henikoff. 2000. Targeting induced local lesions IN genomes (TILLING) for plant functional genomics. Plant Physiol. 123 : 439-442.

Nam, Y.-W., R. V. Penmetsa, G. Endre, P. Uribe, D. Kim, and D. R. Cook. 1999. Construction of a bacterial artificial chromosome library of *Medicago truncatula* and identification of clones for ethylene responsive genes. Theor. Appl. Genet. 98 : 638-646.

Neff, M. M., J. D. Neff, J. Chory, and A. E. Pepper. 1998. dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. Plant J. 14 : 387-392.

Park, S.-Y. and Y.-W. Nam. 2006. Construction of bacterial artificial chromosome library containing large *Bam*HI genomic fragments from *Medicago truncatula* and identification of clones linked to hyper-nodulating genes. J. Microbiol. Biotechnol. 16 : 256-263.

Penmetsa, R. V. and D. R. Cook. 1997. A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. Science 275 : 527-530.

Perry, J. A., T. L. Wang, T. J. Welham, S. Gardner, J. M. Pike, S. Yoshida, and M. Parniske. 2003. A TILLING reverse genetics tool and a web-accessible collection of mutants of the legume *Lotus japonicus*. Plant Physiol. 131 : 866-871.

Seki, M., A. Kamei, K. Yamaguchi-Shinozaki, and K. Shinozaki. 2003. Molecular responses to drought, salinity and frost: common and different paths for plant protection. Curr. Opin. Biotechnol. 14 : 194-199.

Song, K.-H. and Y.-W. Nam. 2005. Genomic organization and differential expression of two polygalacturonase-inhibiting protein genes from *Medicago truncatula*. J. Plant Biol. 48 : 467-478.

Strizhov, N., E. Abraham, L. Okresz, S. Blickling, A. Zilberstein, J. Schell, C. Koncz, and L. Szabados. 1997. Differential expression of two *P5CS* genes controlling proline accumulation during salt-stress requires ABA and is regulated by *ABA1*, *ABI1* and *AXR2* in *Arabidopsis*. Plant J. 12 : 557-569.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22 : 4673-4680.

Till, B. J., C. Burtner, L. Comai, and S. Henikoff. 2004. Mismatch cleavage by single-strand specific nucleases. Nucleic Acids Res. 32 : 2632-2641.

Till, B. J., T. Zerr, L. Comai, and S. Henikoff. 2006. A protocol for TILLING and Ecotilling in plants and animals. Nat. Protocols 1 : 2465-2477.

Trieu, A. T., S. H. Burleigh, I. V. Kardailsky, I. E. Maldonado-Mendoza, W. K. Versaw, L. A. Blaylock, H. Shin, T. J. Chiou, H. Katagi, G. R. Dewbre, D. Weigel, and M. J. Harrison. 2000. Transformation of *Medicago truncatula* via infiltration of seedlings or flowering plants with *Agrobacterium*. Plant J. 22: 531-541.

Troll, W. and J. Lindsley. 1955. A photometric method for the determination of proline. J. Biol. Chem. 215 : 655-660.

VandenBosch, K. A. and G. Stacey. 2003. Summaries of legume genomics projects from around the globe. Community resources for crops and models. Plant Physiol. 131 : 840-865.

Yoshiba, Y., T. Kiyosue, T. Katagiri, H. Ueda, T. Mizoguchi, K. Yamaguchi-Shinozaki, K. Wada, Y. Harada, and K. Shinozaki. 1995. Correlation between the induction of a gene for Δ^1-pyrroline-5-carboxylate synthetase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. Plant J. 7 : 751-760.

Zhu, H. Y., D. J. Kim, J. M. Baek, H. K. Choi, L. Ellis, H. Kuester, W. R. McCombie, H. M. Peng, and D. R. Cook. 2003. Syntenic relationships between *Medicago truncatula* and *Arabidopsis thaliana* reveal extensive divergence of genome organization. Plant Physiol. 131 : 1018-1026.