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Map-based cloning of *chloronerva*, a gene involved in iron uptake of higher plants encoding nicotianamine synthase

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ABSTRACT **The uptake of iron in plants is a highly regulated process that is induced on iron starvation. In tomato, the mutant *chloronerva* exhibits constitutive expression of iron uptake responses and intercostal chlorosis. Biochemically, *chloronerva* is an auxotroph for nicotianamine, a key polyamine in plant iron uptake metabolism. The *chloronerva* gene has been fine-mapped onto the long arm of chromosome 1 in a large segregating tomato population and yeast artificial chromosome clones encompassing the region were isolated by using flanking markers. A cosmid contig containing the *chloronerva* gene was established, and complementing cosmids were identified by transformation into the mutant. The *chloronerva* transcript was identified by cDNA isolation using the complementing cosmids. The gene encodes a unique protein of 35 kDa. The mutant harbors a single base change compared with the wild type. Based on enzyme activity and sequence similarity to the coding DNA sequence of the purified barley enzyme the *chloronerva* gene encodes the enzyme nicotianamine synthase.**

Iron is an essential micronutrient for all living organisms. Under aerobic conditions, iron is almost exclusively present in its oxidized form (Fe^{3+}). Ferric iron has an extremely low solubility and is not readily available to plants in the soil. Thus, plants have developed sophisticated and tightly regulated mechanisms for the acquisition of iron from soil (1, 2). All higher plants, except the Gramineae, use an iron uptake mechanism termed strategy I. The main features of strategy I iron deficiency responses are inducible proton extrusion into the rhizosphere to increase the solubility of Fe^{3+} and the induction of a root-specific iron reductase, which reduces Fe^{3+} to Fe^{2+} at the root surface (3–5).

The *chloronerva* mutant of tomato (*Lycopersicon esculentum*) was identified as a spontaneous mutation in the cultivar Bonner Beste. It possesses a puzzling phenotype showing typical symptoms of iron deficiency, including intercostal chlorosis in young leaves. On the other hand, its iron uptake mechanisms, including proton extrusion and reductase activity, are constitutively expressed, and the mutant accumulates more iron than wild type in all tissues. Grafting the *chloronerva* mutant onto wild type or vice versa normalizes the mutant phenotype, indicating that the lack of a transportable substance is responsible for the mutant phenotype (6). This substance has been purified and identified as the polyamine nicotianamine ((2S:3′:2′S)-*N*-[*N*-{3-amino-3-carboxypropyl}-3-amino-3-carboxypropyl]-azetidine-2-carboxylic acid) (7). Exogenous application of nicotianamine is able to revert the mutant to wild type. Biochemically, nicotianamine is directly synthesized by the condensation of three molecules of S-adenosyl methionine (activated methionine) (8). Based on enzymological evidence, the *chloronerva* mutant is unable to synthesize nicotianamine and lacks the necessary enzyme activity (9).

Nicotianamine plays a central role in iron uptake mechanisms of all plants. In strategy II plants (Gramineae), it is the first intermediate in the synthesis of the phytosiderophores of the mugineic acid type, which mediate the uptake of Fe^{3+}/phytosiderophore complexes into plant roots (10). Its function in strategy I plants is less clear but also of central importance as illustrated by the *chloronerva* mutant. It has been suggested that in strategy I plants nicotianamine is able to form more stable complexes with Fe^{2+} than with Fe^{3+}, serves as a sensor for the physiological iron status within a plant, and/or might be involved in the transport of iron (11, 12). The requirement of a Fe^{2+}/nicotianamine complex in the sensing or transport of iron in the plant would explain the induction of iron deficiency responses in the *chloronerva* mutant because a lack of nicotianamine would be equivalent to iron deficiency.

To investigate whether the *chloronerva* mutation is indeed involved in the synthesis of nicotianamine or, as it has been suggested by others, is a central regulator of iron deficiency responses (13), we have isolated the responsible gene by a map-based cloning approach. The advantage of a map-based cloning approach is that it does not require previous information about the gene product, and tomato is one of the plants for which such an approach is feasible (14). We demonstrate that the *chloronerva* gene encodes nicotianamine synthase (EC 2.5.1.43) based on enzyme activity and homology to the purified and cloned barley enzyme.

MATERIALS AND METHODS

Plant Material and Genetic Mapping. All plant material and genetic mapping procedures have been described (15). For fine mapping of the *chloronerva* gene, the population derived from the cross *chloronerva* x LA716 has been expanded from the previously described 547 F2 plants to 816 F2 plants.

Yeast Artificial Chromosome (YAC) Isolation and Inverse PCR. The flanking markers CT67 and CT224 were used for the screening of a tomato YAC library (16), and the screening was performed by using a PCR protocol with primers derived from those two markers (17). Purification, characterization, pulsed field gel electrophoresis, and end isolation by inverse PCR from YACs were performed according to standard procedures (18). Inverse PCR products from YAC ends were cloned into the pCRII vector (Invitrogen) and sequenced.

Cosmid Isolation and Contig Construction. The YAC ends flanking the gene were used for the isolation of cosmids from two cosmid libraries containing tomato DNA from the varieties Mogeor and Hero. The cosmid libraries were constructed in the transformation vector 04541 in *Escherichia coli* strain

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Abbreviations: YAC, yeast artificial chromosome; cM, centimorgan.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AJ242045).

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XL1 Blue MRF′ Kan (19). Screening of the libraries was performed in a first step by PCR on 192 pools from each library. Each pool represented approximately 1,000 independent clones. Several thousand clones from each positive pool were plated onto agar plates and subsequently screened by colony hybridization using the respective radio-labeled probe. Isolated cosmid clones were purified (Qiagen, Chatsworth, CA), and the ends of the inserts were sequenced on an ABI 377 DNA sequencer according to standard procedures (Applied Biosystems). New primers for cosmid walking were designed from the end sequences, and after mapping onto the recombinant plants they were used to rescreen the cosmid libraries as described above. This procedure was repeated until the entire region between the two flanking markers was isolated in overlapping cosmid clones.

Tomato Transformation. Cosmid clones were isolated from *E. coli* and transferred into *Agrobacterium tumefaciens* LBA 4404 by electroporation. After confirmation that no deletion events occurred in *Agrobacterium*, transformation into the *chloronerva* mutant was performed according to the procedure described in Ling *et al.* (20), and transformed tomato plants were confirmed by PCR.

Transcript Isolation and Characterization. For transcript isolation, complementing cosmids were used for the screening of several cDNA libraries in Lambda gt10 (21) derived from tomato leaf and root tissue. The cosmid inserts were excised with *Xba*I and *Xho*I and purified (GeneClean, BIO 101, Vista, CA). Plaque hybridization was performed with these probes, and positive phages were purified to homogeneity. cDNA inserts were amplified by using gt10 primers and directly sequenced by primer walking. For genomic sequencing, the entire *chloronerva* gene was amplified from DNA of *chloronerva* and the wild-type lines Bonner Beste and Moneymaker by using the primers GTA AGA TTA ACA AAA GGC CTC and TTA CAA TTC CAA GAA AAG AAA ACA. Both strands of the genomic fragments were sequenced by using the same primers as for the cDNA clones. Sequence and protein comparisons were performed by using the National Institutes of Health BLAST server and DNAsis (Hitachi, Tokyo).

Expression in *E. coli* and Enzyme Activity. The complete genomic sequence of the wild-type and mutant *chloronerva* gene was amplified by using primers with appropriate restriction sites. The PCR products were cloned into the expression vector pET12a (Novagen) and transformed into *E. coli* strain DH5alpha. After confirmation of the obtained plasmids by DNA sequencing, they were transformed into the expression strain *E. coli* 174 (DE3). Expression of the *chloronerva* protein was induced as described by the manufacturer of the plasmid. Nicotianamine synthase activity was assayed in sonicated bacterial extracts (9, 22) by using S-adenosyl-L-[carboxyl-^{14}C] methionine. Reaction products were applied to TLC plates (Fluka) and separated with 1-propanol/water (7:8). The synthesized ^{14}C-nicotianamine was detected by using a PhosphorImager (Fuji BAS 2000).

RESULTS

Fine Mapping of the *chloronerva* Gene. The *chloronerva* gene previously was mapped to the long arm of chromosome 1 of tomato (15) by using the mapping population *chloronerva* x LA716 (*L. pennellii*). For the map-based isolation, the population was expanded to 816 phenotypically scored plants. By

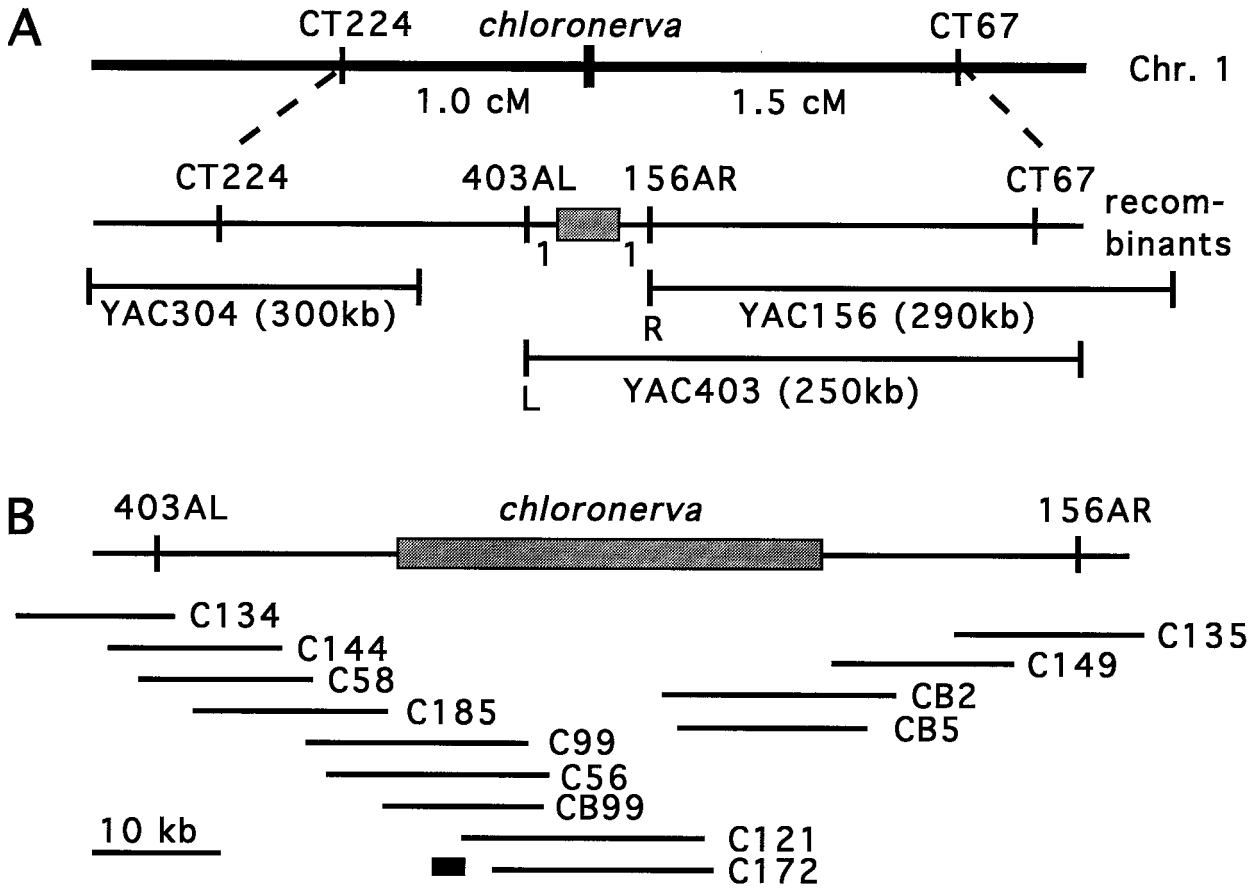

FIG. 1. Map-based cloning of the *chloronerva* gene. (*A*) Genetic fine mapping of the *chloronerva* region and details of the YAC characterization are shown. Note that the genetic distance between CT 67 and the *chloronerva* gene is 1.5 cM. (*B*) Details of the cosmid contig encompassing the *chloronerva* region are shown. Cosmids C99, C56, and CB99 are able to complement the mutant after transformation. The position of the *chloronerva* gene is shown by a black box. Cosmid C121 contains only an incomplete piece of the gene.

using available restriction fragment length polymorphism (RFLP) markers in the region, the *chloronerva* gene was flanked by the RFLP marker CT224 at a distance of 1.0 centimorgan (cM) on the one side and by RFLP marker CT67 at a distance of 1.5 cM on the other side. A chromosome walk was initiated toward the *chloronerva* gene by using a large insert library of tomato in YACs. Several YACs were isolated for each flanking marker (Fig. 1*A*). The ends of those YACs were purified by inverse PCR and mapped as single copy probes relative to the *chloronerva* gene. Although the marker CT67 was separated by 1.5 cM from the *chloronerva* gene, the ends of two YACs isolated with this marker mapped adjacent to the gene. The YAC end 403L could be placed one recombination event left of the gene whereas another YAC end (156AR) was located one recombination event to the right of the gene. With insert sizes of 290 kb (YAC156) and 250 kb (YAC403), these data demonstrated that the ratio of genetic to physical distance in the *chloronerva* region is much lower (1 cM/160 kb) than the average value (1 cM/700 kb) for the tomato genome (23), indicating that the *chloronerva* region is highly active in recombination and the physical distance between the two YAC ends flanking the *chloronerva* gene is likely to be quite small in terms of kb.

Construction of a Cosmid Contig for the *chloronerva* Region. By using the two flanking YAC ends as starting points, cosmid libraries of tomato in the plant transformation vector 04541 were screened, and the construction of a cosmid contig for the entire region between those two markers was initiated. Three walking steps from each side were necessary to cover the entire region, resulting in a contig of approximately 75 kb. After completion of the walk and the localization of the two recombination events in the contig (Fig. 1*B*), cosmids from the region cosegregating with the *chloronerva* gene were transferred into mutant plants by using *Agrobacterium*-mediated transformation. Three cosmids were identified by transformation into the *chloronerva* mutant, which complemented the mutant to wild type based on morphological (Fig. 2) and physiological criteria (15). For each cosmid more than 10 complemented plants were obtained. Physical mapping of the cosmids by restriction digestion revealed a region unique to the three complementing

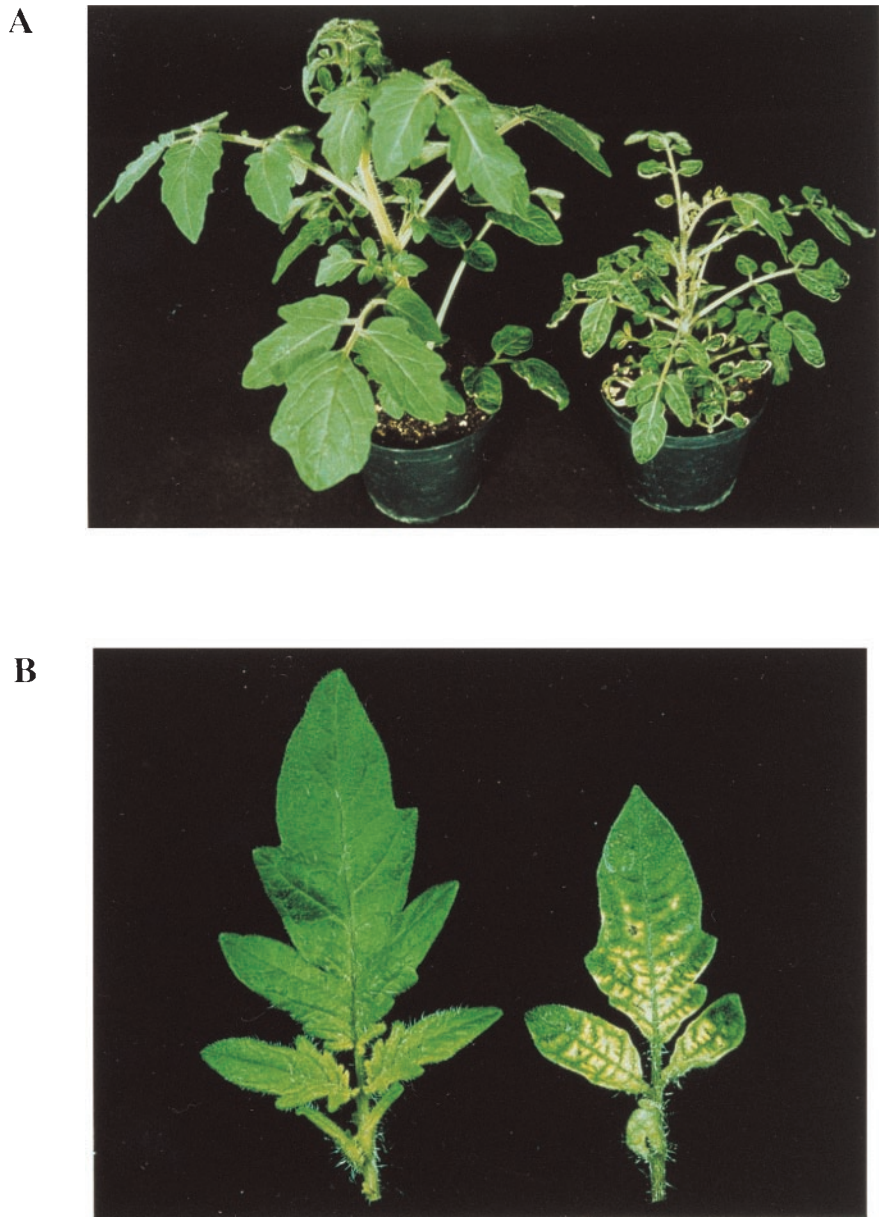

FIG. 2. Complementation of the *chloronerva* mutant. (A) (*Left*) A *chloronerva* plant that has been complemented by transformation with cosmid C99. (*Right*) An untransformed mutant plant. (*B*) Typical leaf of the complemented plant shown in *A* (*Left*) compared with a mutant leaf showing intercostal chlorosis (*Right*).

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          9          18          27          36          45          54
ATT TTT TAC AAT TCC AAG AAA AGA AAA CAA TTT GGT CAT AGT GTC GAC ATG GTG
                                                          M   V

         63          72          81          90          99         108
TGC CCA AAT AGC AAT CCA GTA GTA GAA AAA GTA TGT GAA TTA TAT GAA CAA ATT
 C   P   N   S   N   P   V   V   E   K   V   C   E   L   Y   E   Q   I

        117         126         135         144         153         162
TCA AGA TTG GAG AAC CTT AGC CCT TCC AAA GAT GTC AAC GTA TTG TTC ACA GAT
 S   R   L   E   N   L   S   P   S   K   D   V   N   V   L   F   T   D

        171         180         189         198         207         216
CTT GTC CAC ACG TGC ATG CCT CCT AAT CCC ATT GAT GTC TCT AAG CTC TGT CAA
 L   V   H   T   C   M   P   P   N   P   I   D   V   S   K   L   C   Q

        225         234         243         252         261         270
AAA ATT CAA GAA ATT AGG TCT CAT CTC ATC AAA CTT TGT GGT CAA GCT GAG GGA
 K   I   Q   E   I   R   S   H   L   I   K   L   C   G   Q   A   E   G

        279         288         297         306         315         324
CTT TTA GAG TCA CAC TTT TCT AAA ATT CTT TCC TCC TAT GAA AAC CCC CTT CAA
 L   L   E   S   H   F   S   K   I   L   S   S   Y   E   N   P   L   Q

        333         342         351         360         369         378
CAT CTT CAC ATT TTC CCA TAT TTT GAC AAT TAC ATC AAA CTC AGC TTA CTT GAG
 H   L   H   I   F   P   Y   F   D   N   Y   I   K   L   S   L   L   E

        387         396         405         414         423         432
TAC AAC ATC CTT ACT AAA AAC ACA ACA AAT ATC CCT AAA AAA ATT GCA TTT ATT
 Y   N   I   L   T   K   N   T   T   N   I   P   K   K   I   A   F   I

        441         450         459         468         477         486
GGA TCA GGC CCA CTA CCA CTT ACC TCA CTT GTT TTA GCT ACC AAA CAT CTT AAA
 G   S   G   P   L   P   L   T   S   L   V   L   A   T   K   H   L   K

        495         504         513         522         531         540
ACC ACT TGT TTT CAC AAC TAT GAC ATT GAT GTG GAT GCT AAT TTC ATG GCG TCC
 T   T   C   F   H   N   Y   D   I   D   V   D   A   N   F   M   A   S

        549         558         567         576         585         594
GCC CTT GTG GCG GCC GAT CCA GAC ATG TCC AGC CGT ATG ACT TTT CAT ACG GCT
 A   L   V   A   A   D   P   D   M   S   S   R   M   T   F   H   T   A

        603         612         621         630         639         648
GAC GTC ATG GAT GTA ACG TGT GCC TTG AAA GAC TAC GAT GTA GTC TTT CTG GCC
 D   V   M   D   V   T   C   A   L   K   D   Y   D   V   V   F   L   A

        657         666         675         684         693         702
GCG TTA GTT GGT ATG GAC AAA GAG GAT AAA GTT AAG GTG GTT GAT CAT CTA GCT
 A   L   V   G   M   D   K   E   D   K   V   K   V   V   D   H   L   A

        711         720         729         738         747         756
AAA TAC ATG TCT CCA GGG GCT ACC CTG ATG CTT AGA AGT GCA CAT GGT GCA CGT
 K   Y   M   S   P   G   A   T   L   M   L   R   S   A   H   G   A   R

        765         774         783         792         801         810
GCT TTT CTA TAC CCT GTC CTA GAT CCT CGG GAT CTA CGA GGA TTT GAG GTA CTA
 A   F   L   Y   P   V   L   D   P   R   D   L   R   G   F   E   V   L
    TCT chloronerva
     S

        819         828         837         846         855         864
TCG GTG TAC CAT CCT ACA GAT GAA GTG ATC AAT TCT GTA ATA ATT GCA AGA AAA
 S   V   Y   H   P   T   D   E   V   I   N   S   V   I   I   A   R   K

        873         882         891         900         909         918
TTG CCA GTT CCT AGT GTT CCA CTA CTT GAT GGA TTG GGT GCC TAT GTG TTA CCT
 L   P   V   P   S   V   P   L   L   D   G   L   G   A   Y   V   L   P

        927         936         945         954         963         972
AGC AAA TGT GCT TGT GCT GAG ATT CAT GCT TTC AAT CCA CTC AAT AAG ATG AAT
 S   K   C   A   C   A   E   I   H   A   F   N   P   L   N   K   M   N

        981         990         999        1008        1017        1026
CTG GTT GAA GAA TTT GCT CTG GAG GAG TGA GTG AGA TTT ATG TCT TGT GTT ATG
 L   V   E   E   F   A   L   E   E   *

       1035        1044        1053        1062        1071        1080
TTT CAA TAA TAA TAT TAC TGG AGC ACT TCC ATT TTT ATT GTA ATT TTG TAT CCC

       1089        1098        1107        1116        1125        1134
TAA CTG TTT TAT CAG TGT GTC CTA TTT GTG TGT CTC AAA CTA CAA GAA AAA AGA

       1143        1152        1161        1170        1179        1188
AAA AGG CAT GAG GCC TTT TGT TAA TCT TAC AAA TTT TAT CTA ATA TCT CGT GCC
CA
```

FIG. 3. DNA sequence and protein sequence of the *chloronerva* gene. The DNA sequence of the entire transcript and the amino acid sequence of the coding sequence of the *chloronerva* gene is shown for the cultivars Bonner Beste and Moneymaker. A single base-pair exchange (T to C) at nucleic acid position 761 (amino acid position 238) creates the substitution of a phenylalanine by a serine in the *chloronerva* mutant.

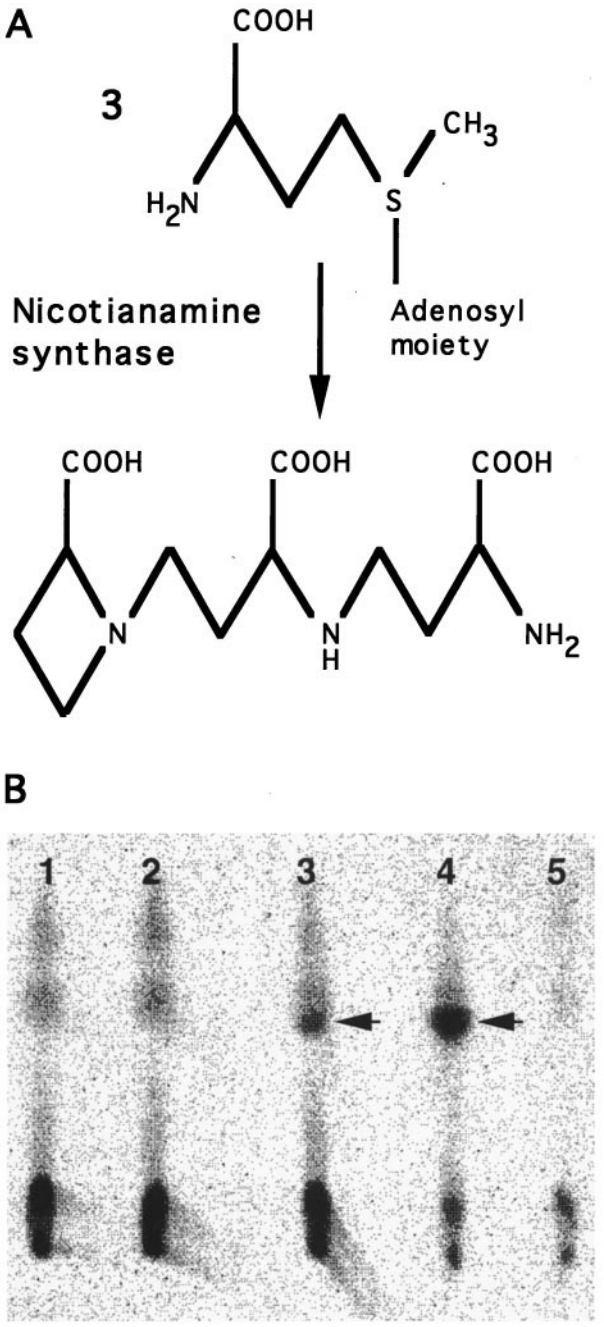

FIG. 4. Nicotianamine synthase activity after expression in *E. coli*. (*A*) Nicotianamine is synthesized by nicotianamine synthase by using three molecules of S-adenosyl methionine. (*B*) Nicotianamine synthase activity was assayed by using radio-labeled S-adenosyl methionine and TLC. Extracts from recombinant *E. coli* strains carrying the mutant allele from tomato (lanes 1 and 2) and the wild-type allele from tomato (lane 3) were used. Lane 4, nicotianamine synthase activity of the cloned barley nicotianamine synthase gene. Lane 5, extracts from an *E. coli* strain carrying only the cloning vector. The position of the nicotianamine product is indicated by arrows.

cosmids of approximately 5–6 kb. Thus, the *chloronerva* gene must be located in this interval. In parallel, cosmids spanning the entire 35-kb region cosegregating with the *chloronerva* gene were used for the screening of cDNA libraries from tomato leaves and roots. A single cDNA class was isolated. By mapping onto the cosmid clones, this cDNA could be delimited to the region shared by the three complementing cosmids, indicating that this gene is a candidate for the *chloronerva* gene.

Characterization of the *chloronerva* Gene. Northern hybridization with the isolated cDNA candidate for the *chloronerva* gene revealed a transcript size of approximately 1.2 kb in roots and leaves of wild-type tomato. The gene is expressed in leaves and roots of tomato independent of the iron status (data not shown). Several full-length cDNA clones with the same transcription start point were isolated from the leaf cDNA library and sequenced (Fig. 3). The length of the full-length transcript is 1,190 bases and in agreement with the predicted size based on the Northern hybridization experiments. PCR analysis of genomic tomato DNA using primers from the 5′ and 3′ flanking untranslated region revealed that the gene does not contain introns within the coding sequence. Genomic South-

ern hybridization demonstrated that the gene is present in the tomato genome as single copy even under reduced stringency conditions (50°C). The predicted size of the encoded protein is approximately 35,000 Da. Because *chloronerva* is a spontaneous mutant derived from the cultivar Bonner Beste, both coding sequences were isolated by PCR and sequenced. A comparison of the two sequences with the unrelated standard tomato line Moneymaker revealed that Bonner Beste and Moneymaker had precisely the same DNA sequence within the transcribed region. In the *chloronerva* mutant a single transition (T to C) was found at amino acid 238 (Fig. 3). This mutation converts a phenylalanine codon (TTT) into a serine codon (TCT).

Detection of Nicotianamine Synthase Activity After Expression in *E. coli*. To demonstrate that the chloronerva gene is indeed nicotianamine synthase, the coding sequence was cloned into an expression vector. The expression of the recombinant protein in *E. coli* revealed nicotianamine synthase activity, and the product nicotianamine is detected with the wild-type *chloronerva* gene (Fig. 4) as well as with the recently cloned nictotianamine synthase from barley (A. Herbik, H.-P. Mock, G.K., A. Czihal, J. Thielmann, U. W. Stephan, and H.B., unpublished work). The enzyme activity of the tomato enzyme appears to be weaker than of the barley enzyme. No detectable nicotianamine synthase activity is found after expressing the mutant *chloronerva* gene in *E. coli,* which indicates that the point mutation identified in the *chloronerva* mutant reduces significantly or abolishes the enzyme activity and that the mutated amino acid probably plays a crucial role for the proper function of the enzyme.

Sequence Comparison of the *chloronerva* Gene. A search with the *chloronerva* gene sequence in the DNA and protein databases revealed no significant homology to known genes from other organisms. Only weak homology to an unknown protein of *Methanobacterium thermoautothropicum* was found (BLAST score 83; 4.8×10^{-5}). This finding indicated that the *chloronerva* gene encodes a type of protein not previously found in bacteria, plants, or animals. Two sequences with unknown function showed strong homology with the *chloronerva* gene (Fig. 5). Both genes are derived from the *Arabidopsis* sequencing project, and they are located on bacterial artificial chromosomes from chromosomes 1 and 5, respectively. The two *Arabidopsis* genes encode proteins of nearly the same size as the *chloronerva* gene. A detailed alignment of the sequences revealed that the percentage of identical amino acids with the two *Arabidopsis* sequences is 59% (sequence on chromosome 1) and 57% (sequence on chromosome 5), respectively.

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NATOMATO  MVCPNS--NPVVEKVCELYEQISRLENLSPSKDVNVLFTDLVHTCMPPNP-IDVSKLCQKIQE
NABARLEY  MDAQNKEVDALVQKITGLHAAIAKLPSLSPSPDVDALFTDLVTACVPPSP-VDVTKLGSEAQE
ARA1      MGCQD---EQLVQTICDLYEKISKLESLKPSEDVNILFKQLVSTCIPPNPNIDVTKMCDRVQE
ARA5      MACQN---NLVVKQIIDLYDQISKLKSLKPSKNVDTLFGQLVSTCLPTDTNIDVTNMCEEVKD
          *   #      * #    *   *  *  *  *#** #*  **##** * *# #  ** ##    ##

NATOMATO  IRSHLIKLCGQAEGLLESHFSKILSSYE---NPLQHLHIFPYFDNYIKLSLLEYNILTKN-TT
NABARLEY  MREGLIRLCSEAEGKLEAHYSDMLAAF--D-NPLDHLGMFPYYSNYINLSKLEYELLARYVPG
ARA1      IRLNLIKICGLAEGHLENHFSSILTSYQ-D-NPLHHLNIFPYYNNYLKLGKLEFDLLEQNLNG
ARA5      MRANLIKLCGEAEGYLEQHFSTILGSLQEDQNPLDHLHIFPYYSNYLKLGKLEFDLLSQH-SS
           *  ** #*  *** ** * *  *       *** **  ***  **# *# **#  *

NATOMATO  -NIPKKIAFIGSGPLPLTSLVLATKHLKTTCFHNYDIDVDANFMASALVAADPDMSSRMTFHT
NABARLEY  RHRPARVAFIGSGPLPFSSYVLAARHLPDAMFDNYDLCSAANDRASKLFRADKDVGARMSFHT
ARA1      -FVPKSVAFIGSGPLPLTSIVLASFHLKDTIFHNFDIDPSANSLASLLVSSDPDISQRMFFHT
ARA5      -HVPTKIAFVGSGPMPLTSIVLAKFHLPNTTFHNFDIDSHANTLASNLVSRDPDLSKRMIFHT
             *   **#****#*  * ***  **    * *#*    **  ** *  #* *   ** ***

NATOMATO  ADVMDVTCALKDYDVVFLAALVGMDKEDKVKVVDHLAKYMSPGATLMLRSAHGARAFLYPVLD
NABARLEY  ADVADLTGELAAYDVVFLAALVGMAAEDKTKVIAHLGAHMADGAALVVRSAHGHVGFLYPIVD
ARA1      VDIMDVTESLKSFDVVFLAALVGMNKEEKVKVIEHLQKHMAPGAVLMLRSAHGPRAFLYPIVE
ARA5      TDVLNATEGLDQYDVVFLAALVGMDKESKVKAIEHLEKHMAPGAVLMLRSAHALRAFLYPIVD
          #*# # *  *  #***********  *#* *#  **   *  ** *  ****#   ****  #

NATOMATO  PRDL--RGFEVLSVYHPTDEVINSVIIARKL-PVPSVPLLDGLGA-------YVLPSKCACAE
NABARLEY  PQDIGRGGFEVLAVCHPDDDVVNSVIIAHKSKDVHANERPNGRGGQYARGAVPVVSPPCRFGE
ARA1      PCDL--QGFEVLSIYHPTDDVINSVVISKKH-PVVSIGNVGGPNS-------CLLKP-CNCSK
ARA5      SSDL--KGFQLLTIYHPTDDVVNSVVIARKL-GGPTTPGVNGTRG-------CMFMP-CNCSK
          # *    **### # ** * * ***#*# *   #       * #         #    *   #

NATOMATO  IHA-FNPLNKMNLVEEFALEE
NABARLEY  MVADVTHKR-----EEFTNAEVAF
ARA1      THAKMN---KNMMIEEFGAREEQLS
ARA5      IHAIMNNRGKKNMIEEFSAIE
             *   *        ***   *
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FIG. 5. Comparison of the *chloronerva* gene to other DNA sequences. The alignment of the *chloronerva* gene (NATOMATO) to the purified nicotianamine synthase (NABARLEY) from barley is displayed. Furthermore, a comparison of the two genomic sequences (ARA1 and ARA5) from the *A. thaliana* genome on chromosome 1 (GenBank accession no. AC003114) and 5 (GenBank accession no. AB005245), respectively, is shown. Amino acids matching in all four proteins are marked by ∗. Additional amino acids shared between the *chloronerva* gene and nicotianamine synthase of barley are marked by #. The phenylalanine that has been changed to a serine in the *chloronerva* mutant is shown in bold. Note that this amino acid is highly conserved in all four genes.

A sequence comparison with the nicotinamine synthase from barley (A. Herbik, H.-P. Mock, G.K., A. Czihal, J. Thielmann, U. W. Stephan, and H.B., unpublished work) also indicates that *chloronerva* is homologous to this gene. The *chloronerva* gene and nicotianamine synthase from barley share 51% of all amino acids. Conserved amino acid exchanges account for an additional 25% of the amino acids. Analyzing all four sequences together showed that the mutation found in the *chloronerva* sequence is within a region of the protein that is highly conserved between all four genes, again suggesting that a functionally significant region of the protein has been altered.

DISCUSSION

Biochemical evidence and enzyme assays have shown previously that the *chloronerva* mutant lacks the polyamine nicotianamine, which suggested that the synthesis of nicotianamine from the starting substrate S-adenosyl methionine via the enzyme nicotianamine synthase is blocked. Although most of the biochemical evidence favors this hypothesis, the pleiotrophic phenotype of the *chloronerva* mutant and some inhibitor studies have suggested that the *chloronerva* gene might not encode nicotianamine synthase but another more central product that controls the synthesis of nicotianamine and other iron deficiency responses under the possible influence of phytohormones (13).

To clarify this question and to get a more detailed insight into the function of the *chloronerva* gene in iron uptake mechanisms, we have isolated the *chloronerva* gene by a map-based cloning approach. The advantage of a map-based cloning approach is that it does not require any *a priori* information about the involved gene (14). Furthermore, the observed nicotianamine synthase activity in wild-type tomato is low and not inducible by iron deficiency, and the enzyme is difficult to purify by biochemical means (8, 9).

The evidence that the isolated cDNA is indeed the *chloronerva* gene is based on several arguments.

First, the identified gene cosegregates perfectly with the *chloronerva* gene, and it is located within the complementing region of approximately 5–6 kb in the cosmid contig. Second, this cDNA is the only transcript isolated within the complementing region.

That the function of the *chloronerva* gene is indeed in the synthesis of nicotianamine is confirmed by the fact that the wild-type *chloronerva* gene has nicotianamine synthase activity and that it is the tomato homologue of nicotianamine synthase of barley which was recently isolated (A. Herbik, H.-P. Mock, G.K., A. Czihal, J. Thielmann, U. W. Stephan, and H.B., unpublished work). The enzyme was purified from iron-deficient barley roots, and it was demonstrated that the expressed protein also has nicotianamine synthase activity. The active form of nicotianamine synthase is most likely homomultimeric. Fifty-one percent of all amino acids of the *chloronerva* gene and nicotianamine synthase of barley are identical, and an additional 25% of the amino acids are conserved exchanges. Together with the same enzyme activity, this finding indicates that they are indeed orthologous genes. The tomato enzyme, as the barley nicotianamine synthase, lack

motifs similar to other enzymes that use S-adenosyl methionine as substrate, suggesting that the enzymatic mechanism is different from these enzymes (24).

Furthermore, a point mutation could be detected within the candidate cDNA between wild-type Bonner Beste and the *chloronerva* mutant. This mutation changes an amino acid that is highly conserved among the related genes. It also abolishes nicotianamine synthase activity in enzyme assays.

Nicotianamine synthase is a unique protein with no known homologues in eukaryotes other than plants, which is not unexpected because nicotianamine is found only in plants and not in microorganisms or animals (25, 26). Two related genes are found only in *Arabidopsis thaliana* in the database search. The function of those genes is at present not clear because the sequence data are derived from genomic sequencing and no physiological description or mutants are available. The high sequence homology suggests that one of those genes might be a homologue to the nicotianamine synthase sequence described here. Further research will be necessary to elucidate their role by enzyme assays or complementation of the *chloronerva* mutant by using these genes.

The isolation of the *chloronerva* gene and the finding that this gene is homologous to a nicotianamine synthase gene in barley confirms the previous data suggesting that nicotianamine has a central function in strategy I iron metabolism (11, 12). The availability of the gene will permit a more detailed analysis of the role of nicotianamine in the individual iron deficiency responses. For example, by tissue-specific overexpression of nicotianamine synthase in tomato and a detailed investigation in which way this changes iron deficiency responses, it will be possible to determine in what way nicotianamine actually is involved in the sensing of the iron status of strategy I plants.

Note Added in Proof. The isolation and DNA sequence of nicotianamine synthase from barley recently has been published by Higuchi *et al.* (27).

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