Expression of a *Brassica napus* Malate Synthase Gene in Transgenic Tomato Plants during the Transition from Late Embryogeny to Germination

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ABSTRACT

To study gene regulation during the transition from late embryogeny to germination, we have analyzed the expression of a gene encoding the glyoxylate cycle enzyme malate synthase in transgenic tomato (*Lycopersicon esculentum*) plants. We have shown that although there are at least four classes of malate synthase genes in *Brassica napus* L., one gene is expressed at a high level during both late embryogeny and postgermination. Analyses of transgenic tomato plants containing the expressed *B. napus* gene along with 4.7 and 1.0 kilobase pairs of 5' and 3' flanking sequences, respectively, confirmed that a single gene is expressed at both stages of development. Furthermore, localization studies have shown that mRNA encoded by the *B. napus* gene is distributed throughout the tissues of a mature embryo but is not detected in the vascular cylinder of a seedling. We conclude that the sequences required to qualitatively regulate the gene correctly over the plant life cycle are present within the transferred gene and/or flanking regions. Moreover, the malate synthase gene is regulated differently during late embryogeny and postgermination in the developing vascular cylinder.

There are substantial differences in the physiological and morphological processes that occur during late embryogeny and postgermination (reviewed in ref. 2). For example, late embryogeny is characterized by a decline in embryo water potential, a decrease in metabolic rates, chlorophyll loss, and the stabilization of cellular structures in a desiccated state. By contrast, following the rehydration of dry seeds, a rapid increase in metabolic rates occurs, storage reserves including lipids and proteins are mobilized to provide nutrients for the seedling, and growth occurs by both cell expansion and cell division. These changes in large part reflect different patterns of gene expression (6, 10, 15); distinct sets of mRNAs have been shown to accumulate primarily during late embryogeny or during germination and postgermination (5, 18, 19). Moreover, analysis of the specific transcripts produced in dry seed nuclei indicates that the repression of genes expressed during late embryogeny and the activation of genes expressed primarily during postgermination occur after seeds are rehydrated (5). Thus, seed imbibition appears to initiate processes that induce a shift from a late embryonic to a germinative/postgerminative program of gene expression during normal plant development.

To understand the processes that operate during the transition from embryogeny to germination, we are studying the regulation of genes encoding the glyoxylate cycle enzyme malate synthase (E.C. 4.1.3.2) in *Brassica napus* L. Malate synthase functions primarily during seed germination and postgermination by catalyzing a reaction involved in the mobilization of storage lipids (31). As predicted by this role in postgermination, malate synthase mRNA, protein, and enzymatic activity attain maximal levels in the cotyledons of seedlings and are not detected in leaves of a mature plant (4, 12, 27, 29, 32, 33). However, we and others have also shown that malate synthase mRNA and protein accumulate initially during late embryogeny and are stored in dry seeds (4, 26, 32). Because malate synthase accumulates during late embryogeny and postgermination, understanding the mechanisms regulating the genes should provide information about processes involved in controlling the transition from embryogeny to germination. In this regard, we have shown previously that the expression of malate synthase genes is regulated primarily at the transcriptional level (4).

We have identified four classes of malate synthase genes in *B. napus* and obtained the nucleotide sequence of one gene that is highly expressed in both embryos and seedlings. Our studies show that although this single gene is expressed during late embryogeny and postgermination of *B. napus* and transgenic tomato plants, mRNA encoded by the gene are distributed differently in mature embryos and seedlings. The implication of these studies to the regulatory programs that operate during the transition from embryogeny to germination is discussed.

MATERIALS AND METHODS

Plant Material

*Brassica napus* L. (rapid cycling base population, CrGC5) plants and seedlings were grown and embryos were staged as described previously (18). Tomato plants (*Lycopersicon esculentum* cv VF36) were grown under standard greenhouse conditions at the University of California, Davis, California 95616 during the summer months.
conditions. For germination studies, tomato seeds were incubated in the dark at 25°C on moist filter paper in Petri dishes. At specified times after the start of imbibition, seedlings without seed coats were frozen immediately in liquid nitrogen for RNA isolation.

**Nuclear DNA Isolation and DNA Gel Blot Hybridization**

*B. napus* nuclear DNA was isolated and DNA gel blot hybridization experiments were performed as described previously (3). Tomato nuclear DNA was isolated using the hexadecyltrimethylammonium bromide method (9).

**Nuclear DNA Library and Genomic Clone Isolation**

*B. napus* nuclear DNA was partially digested with Sau3AI, and fractionated DNA fragments with an average size of 20 kb\(^2\) were cloned into the *Bam*H I site of the bacteriophage lambda vector Charon 35 (23) using standard protocols (20). Malate synthase genomic clones were isolated from the library using the insert from the malate synthase cDNA clone, pMS19, in plaque hybridization experiments (3).

**DNA Sequencing**

A nested set of deletions of the malate synthase gene was generated by exonuclease III digestion protocols and the gene was sequenced in both directions using methods described previously (3).

**Plant Transformation**

The 8.7 kb XhoI fragment containing the *MS-A* gene (see Figure 1) was inserted in both orientations into the SalI site of the vector pMON200 (13). The constructions were integrated into the Ti plasmid present in *Agrobacterium tumefaciens* strain GV3111 = C58C1 by triparental mating (13). Tomato seeds were germinated aseptically on MS salts (Gibco) containing 3% sucrose, B5 vitamins, 0.8% agar, pH 5.8. Cotyledons of seedlings grown for 14 d were co-cultivated with *A. tumefaciens* as described by McCormick et al. (24). Regenerated plantlets were selected on media containing kanamycin (100 \(\mu\)g/mL), transferred to soil, and grown in the greenhouse.

**RNA Isolation**

Total and polyadenylated RNA were isolated using procedures described previously (18) or using the following modification of these methods. Two hundred to 500 mg of frozen tissue was pulverized in liquid nitrogen and transferred to a polypropylene tube containing 2 mL of extraction buffer (100 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5% SDS, pH 9). One milliliter of phenol saturated with 100 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 9 and prewarmed to 65°C was added, and the mixture was shaken vigorously for 15 min. One milliliter of chloroform:isoamyl alcohol (24:1, v/v) was then added, and the extraction was continued for an addi-

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2 Abbreviations: kb, kilobase(pairs); bp, basepairs; DAI, days after the start of imbibition.
One Malate Synthase Gene Encodes an mRNA that is Prevalent in Both Embryos and Seedlings of *Brassica napus*

We showed previously that at least one class of malate synthase mRNA is present at a high level in seedlings, and hybridization studies at a high criterion predicted that this mRNA is encoded by the *MS-A* gene (3). To confirm this observation, the nucleotide sequence of the *MS-A* gene was obtained and is shown in Figure 3. With the exception of three introns, 155, 240, and 484 bp in size, the sequence of the *MS-A* gene is identical to that of the abundant malate synthase mRNA present in seedlings (3). Because there is significant sequence divergence between the malate synthase genes (3), these results indicate that the *MS-A* gene is expressed at a high level during postgermination. We note that a cucumber malate synthase gene contains three introns that are located at identical positions within the protein coding region (16).

Because malate synthase mRNA accumulates not only during postgermination but also during late embryogeny, we asked whether the same malate synthase gene is expressed in embryos. As shown in Figure 4, mRNAs from dry seeds (DS) which represent transcripts synthesized during late embryogeny and from seedlings (S) grown for 3 DAI were hybridized with genomic clones representing the three malate synthase gene classes that had been labeled to equivalent specific radioactivities. Each genomic clone reacted with the 2.1 kb malate synthase mRNA present in dry seeds and seedlings as well as other mRNAs that may correspond to non-homologous genes present on the genomic clone. Of the three genes, *MS-A* hybridized with greatest intensity to malate synthase mRNA from both embryos and seedlings (compare lanes 1–2 with lanes 3–6). Higher temperature washes of the RNA gel blots confirmed that the malate synthase mRNA present in dry seeds and seedlings share the greatest sequence similarity with the *MS-A* gene (data not shown). The results indicate...
that the same malate synthase gene, MS-A, is expressed at high levels not only during postgermination but also during late embryogenesis. Because the other malate synthase genes cross-hybridize with MS-A, it is unclear whether these other genes are expressed at lower levels.

A Single Malate Synthase Gene is Expressed in Embryos and Seedlings of Transgenic Tomato Plants

The MS-A gene along with 4.7 kb and 1.0 kb of 5′ and 3′ flanking sequences, respectively, was transferred into tomato plants using a vector derived from the Ti plasmid of Agrobacterium tumefaciens to begin investigating the mechanisms regulating its expression. Transgenic plants were obtained using modified Ti plasmids containing the MS-A gene in both orientations relative to vector sequences (see Figure 5, legend). DNA gel blot in Figure 5A show that the six transgenic plants each contain approximately 1–3 copies of the MS-A gene which are not rearranged detectably and that the MS-A cDNA clone does not possess sufficient sequence similarity to react with the endogenous tomato malate synthase gene(s) under the hybridization conditions employed.

To determine whether the B. napus gene is expressed correctly in transgenic tomato plants, we measured MS-A mRNA levels using primer extension assays. Figure 5B shows that transcription of the MS-A gene is initiated at an identical position in B. napus (lane BN) and in the six transgenic tomato plants (lanes T1, T2, 3, 4, 5, and 6). The assay did not measure mRNA(s) encoded by the endogenous tomato gene(s) (lane NT) presumably because it is not complementary to the primer specific for the B. napus gene. Additionally, RNA gel blot experiments showed that 2.1 kb MS-A mRNAs are detected in transgenic tomato seedlings that are identical in size to malate synthase mRNA in B. napus (data not shown). The temporal pattern of MS-A gene expression in one transgenic tomato plant, designated T1, is shown in Figure 6, A and B. MS-A mRNA accumulation is similar to that observed in B. napus; mRNA levels increase then decrease during postgermination. Similar patterns of mRNA accumulation also were observed in plants T5 and T10 (data not shown). These observations were surprising initially because, as shown in Figure 6B, the time course of endogenous malate synthase enzyme activities differ in B. napus and tomato. However, comparison of Figure 6B, C, and D, shows that the levels of mRNA encoded by the B. napus MS-A gene and by the endogenous tomato gene(s) are modulated similarly. Thus, the lag period separating the increase in malate synthase mRNA level and enzyme activity is significantly longer in tomato.

We confirmed the prediction that this single gene from B. napus is also expressed during embryogenesis. Figure 5, B and C, shows that MS-A mRNA is present not only in seedlings grown for 3 DAI, but the gene is also expressed during embryogenesis as indicated by its presence in dry seeds of the
Figure 5. Organization and expression of the malate synthase gene in transgenic tomato plants. A, DNA gel blot analysis of MS-A genes in transgenic tomato plants. Nuclear DNA from six independently derived transgenic plants (T1, T10, T3, T8, T2, and T5) and one non-transformed tomato plant (NT) were digested with the restriction enzyme HindIII, fractionated on a 0.7% agarose gel, blotted to filters, and hybridized with the radioactively labeled malate synthanse cDNA clone pMS1. The MS-A gene and its flanking sequences are present in opposite orientations relative to vector sequences in transgenic plants T1, T10, and T8 (note restriction fragments at 1.5 kb) in T3, T2, and T5 (note high molecular weight restriction fragments). A single copy reconstruction of the cloned MS-A gene is also shown (SC). The autoradiogram shows that each plant contains between one and three copies of the MS-A gene. The probe does not hybridize with the endogenous malate synthase gene(s) of non-transformed tomato (NT) under the hybridization conditions employed. B, Primer extension analysis of MS-A mRNA in transgenic tomato plants. Malate synthase mRNA levels were measured in dry seeds, seedlings grown for 3 DAI, and leaves from each of the transgenic tomato plants using primer extension assays. A primer complementary to MS-A mRNA was annealed with total RNA from dry seeds (30 µg), 3 DAI seedlings (10 µg), or leaves (10 µg) and extended in vitro. Three major extension products that terminate at nucleotides +1, +4, and +5 in the MS-A gene sequence shown in Figure 3 were observed following gel electrophoresis. RNA (3.3 µg) from 3 DAI B. napus seedlings was used as a standard (Bn) for all three sets of experiments. C, Quantitation of MS-A mRNA levels. For each transgenic plant, MS-A mRNA levels in dry seeds (D), seedlings grown for 3 DAI (S), and leaves (L) were determined in primer extension assays similar to those shown in (B). The reported values represent the average of 3–4 independent determinations except for dry seed values for plants T3 and T6 in which 2 determinations were done. Brackets show one standard deviation.
six transgenic plants. MS-A mRNA levels in transgenic plants attain between 30% to 80% of levels present in B. napus seeds and seedlings at comparable stages; this plant-to-plant variability is similar to that observed for the expression of other foreign genes in transgenic plants (21). The orientation of the gene relative to vector sequence in the tomato genome did not appear to influence consistently mRNA levels (compare T1, 10, 8 versus T3, 2, 5). Moreover, we also showed that MS-A mRNA is not detected in young fully expanded leaves of the transgenic plants (<7 × 10⁻²% of total RNA). Thus, although the absolute levels of MS-A mRNA differ in each transgenic plant, the B. napus malate synthase gene appears to be qualitatively regulated correctly.

We asked whether the B. napus malate synthase gene encodes a functional protein in tomato. The cross-reaction of antibodies against B. napus malate synthase with an endogenous tomato protein of similar molecular mass prohibited us from measuring MS-A protein levels directly. However, Table I shows that malate synthase enzyme activity in transgenic seedlings grown for 5 DAI correlates roughly with the level of MS-A mRNA accumulation, suggesting that the B. napus gene product is functional in tomato. By contrast, the activities of another glyoxylate cycle enzyme, isocitrate lyase, is equivalent in the non-transformed and transgenic plants (data not shown).

**Malate Synthase mRNA is Distributed Differently in Mature Embryos and Seedlings**

Our results showed that the malate synthase gene, MS-A, is expressed at a high level during embryogenesis and postgermination in B. napus and transgenic tomato plants. To study further the regulation of this gene, we analyzed its spatial pattern of expression using in situ hybridization protocols. Figure 7A shows a longitudinal section through the axis of a mature B. napus embryo from a dry seed that was hybridized with an antisense RNA probe to MS-A. As indicated by the position of the white silver grains in the dark-field photomicrograph shown in Figure 7B, malate synthase mRNA accumulates throughout the tissues of the axis. By contrast, Figure 7, D and E, shows that malate synthase mRNA is prevalent in the epidermis and ground tissue of the axis of a seedling grown for 1 DAI but is present at a lower level in the vascular cylinder. Figure 7, C and F, shows that a sense RNA probe identical in sequence to MS-A mRNA does not bind significantly to the sections. Thus, malate synthase genes are expressed differently during embryogenesis and postgermination in the developing vascular cylinder.

In principle, the distribution of malate synthase mRNA in B. napus could result from the expression of several members of the gene family (Figs. 1 and 2). Therefore, parallel experiments were performed with transgenic tomato plants containing the MS-A gene. Figure 7, G and H, shows the hybridization of MS-A antisense RNA with a longitudinal section through a dry seed from the transgenic tomato plant T3. MS-A mRNA accumulates throughout the mature embryo, including the procambium, and in the endosperm of the dry seed. The hybridization of a comparable section with a ribosomal RNA probe (Fig. 7, M and N) showed that MS-A mRNA and rRNA are distributed similarly in the dry seed although at different levels (see Fig. 7, legend). In transgenic tomato seedlings grown for 1 DAI, MS-A mRNA accumulates in storage parenchymal, epidermal, and cortical cells, but similar to B. napus, the mRNA is not detected in the vascular cylinder of the seedlings or in the endosperm (Fig. 7, J and K). Identical distribution patterns were observed with seeds and seedlings from transgenic plant T8 (data not shown). The hybridization of a rRNA probe indicates that the vascular cylinder of the seedling and the endosperm contain RNAs that are accessible in the hybridization reaction (Fig. 7, O and P). Figure 7, I and L, shows that the endogenous malate synthase mRNA present in non-transformed tomato dry seeds and seedlings, respectively, do not react with the B. napus probes. These results suggest that during the transition from late embryogenesis to postgermination, a switch in malate synthase gene expression occurs in the procambium/vascular cylinder.

**Table I. Malate Synthase Enzyme Activity in Transgenic Tomato Seedlings**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Enzyme Activity at 5 DAI</th>
<th>Fraction of Total RNA at 3 DAI</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>µmol min⁻¹ mg protein⁻¹</td>
<td>× 10⁶</td>
</tr>
<tr>
<td>Non-transformed</td>
<td>0.12 ± 0.07 (1)ᵇ</td>
<td>ND</td>
</tr>
<tr>
<td>T5</td>
<td>0.19 ± 0.11 (1.5)ᵇ</td>
<td>2.1</td>
</tr>
<tr>
<td>T1</td>
<td>0.22 ± 0.05 (1.8)ᵇ</td>
<td>2.6</td>
</tr>
<tr>
<td>T3</td>
<td>0.42 ± 0.22 (3.4)ᵇ</td>
<td>5.7</td>
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ᵇ Average of three independent determinations.
DISCUSSION

Malate synthase accumulates during late embryogeny and postgermination, two stages of development that are separated by a period of developmental arrest. In *B. napus*, the enzyme is encoded by a relatively small gene family (3) and mRNA accumulation is regulated primarily at the transcriptional level although posttranscriptional processes also appear to be involved (4, 12). Defining the regulatory mechanisms controlling malate synthase gene expression should provide insight into the processes that operate during the transition from embryogy to germination.

One Class of Malate Synthase Genes is Highly Expressed during Late Embryogeny and Postgermination

Our results indicate that the malate synthase gene family consists of at least four classes of genes (Figures 1 and 2). Analyses of nuclear DNA from *B. campestris, B. oleracea*, and monosomic alien chromosome addition lines suggest that each class of malate synthase genes is present in the progenitors of the amphidiploid *B. napus* (25); L. Comai and J.J. Harada, unpublished results. This finding implies that each gene class should consist of two homeologous loci as appears to be the case for MS-C. However, examination of 21 overlapping MS-A genomic clones did not reveal restriction site polymorphisms (data not shown). If there are two distinct MS-A genes, they appear to be highly conserved both within the gene and in flanking regions.

Sequence divergence among the classes of malate synthase genes enabled us to determine that one gene, MS-A, is expressed at high levels in embryos and seedlings of *B. napus* (Figure 4). Expression of the MS-A gene in transgenic tomato plants confirmed that this single gene is expressed at both developmental stages although we have not shown precisely when the gene is activated initially during embryogeny (Figure 5). Unlike other gene families in which alternate members are expressed at different developmental stages, e.g., the glutamine synthase genes of pea and phylalanine ammonium lyase genes of beans (22, 30), malate synthase accumulation during late embryogeny and postgermination does not appear to result exclusively from gene switching processes.

Our experiments do not indicate whether the other malate synthase genes besides MS-A encode low abundance mRNAs present in embryos or seedlings or at other stages of the life cycle. However, we note that the MS-C genomic clones each contain only one restriction fragment, 1.1 kb in size, that reacts with the malate synthase cDNA clone (Figure 1). These 1.1 kb fragments are smaller than the 2.1 kb malate synthase mRNA indicating that the MS-C genes either do not encode functional proteins or that the clones contain only parts of the genes. Furthermore, it is unlikely that the malate synthase gene present on the 4.8 kb HindIII restriction fragment shown in Figure 2 is expressed at high levels in seedlings; all characterized malate synthase cDNA clones from seedlings are derived from the MS-A gene (3).

Malate Synthase Gene Regulation during Late Embryogeny and Germination

Although the vast majority of genes expressed in seedlings are also expressed in embryos (14), distinct sets of genes have been identified that accumulate specifically during late embryogeny or during postgermination (5, 18, 19). Our previous studies implicate events that occur during the rehydration of dry seeds as a temporal switch that separates embryonic from germinative/postgerminative patterns of gene expression (5). These results imply that distinct factors operate in regulating gene expression during late embryogeny and postgermination.

Malate synthase genes as well as several other genes encoding mRNAs that are prevalent in the cotyledons of *B. napus* seedlings are expressed during late embryogeny and postgermination (4, 18). Two simple models may explain the regulation of malate synthase genes (18). First, consistent with the idea that a general switch in gene regulation occurs during seed imbition, different cellular factors may regulate the MS-A gene during late embryogeny and postgermination. The second model proposes that the MS-A gene is regulated by the same factor(s) during both stages of development. This second viewpoint would imply that a distinct regulatory program operates during late embryogeny and postgermination to activate and repress malate synthase and other similarly expressed genes.

To begin to distinguish between these possibilities, we transferred an expressed malate synthase gene from *B. napus* into tomato plants in order to focus exclusively on the expression of one member of the gene family. Several lines of evidence suggest that the *B. napus* gene MS-A is regulated correctly in transgenic tomato plants. First, MS-A mRNA is detected only during the expected stages of the life cycle (Figure 5). Second, similar to *B. napus*, MS-A mRNA levels are modulated during postgermination (Figure 6). Third, MS-A mRNA is localized in transgenic tomato seeds and seedlings as it is in *B. napus* (Figure 7). These results indicate that the *cis*-acting sequences that play a major role in regulating the gene are likely to reside within the gene and/or flanking regions that were transferred into the plants. Similar results showing the expression of a cucumber malate synthase gene in seedlings of transgenic tobacco and petunia plants have been reported recently by Graham et al. (17). Their results suggest that the *cis*-acting sequences regulating the malate synthase gene are localized in the 5' flanking region.

The spatial distributions of *B. napus* malate synthase mRNA in mature embryos and seedlings of transgenic tomato plants provide information relevant to the two models of gene regulation. The presence of malate synthase mRNA in the procambium of mature embryos and its absence from the vascular cylinder of seedlings (Figure 7) demonstrates a temporal switch in malate synthase gene regulation that occurs following germination. The procambial tissue of the mature embryo undergoes extensive differentiation following germination to form the stele of the seedling axis (11). The signals that fail to activate or repress malate synthase genes in the vascular cylinder of seedlings may be generated by histodifferentiation events and/or the depletion of storage lipids from this tissue.

In contrast to the developing vascular cylinder, the similar distribution of malate synthase mRNA in the epidermal and ground tissues of mature embryos and seedlings does not provide support for or against either model of gene regulation. The MS-A gene may be regulated by different mechanisms in embryos and seedlings if the regulatory circuitry of the gene...
is complex. For example, one tissue-specific regulatory element may operate during late embryogenesis while another may function during postgermination. Other genes expressed in plants have been shown to be regulated through combinatorial mechanisms (1). Alternately, the results may indicate simply that the gene is regulated by a common mechanism during late embryogenesis and postgermination in the epidermal and ground tissues. Resolution of these issues will ultimately come with the identification of the cis-acting regulatory sequences and trans-acting factors that regulate this gene.

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