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Full Length Research Paper

Overexpression of a foxtail millet Acetyl-CoA carboxylase gene in maize increases sethoxydim resistance and oil content

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Acetyl-coenzyme A carboxylase (ACCase) plays a crucial role in fatty acid metabolism in plants, catalyzing the carboxylation of acetyl-CoA to produce malonyl-CoA. In grasses, the plastids ACCases are critical determinants of plant sensitivity to herbicide classes cyclohexanediones (CHDs) and aryloxyphenoxy propionates (APPs). We transformed the full-length cDNA of a plastid ACCase gene from foxtail millet (*Setaria italica*) line Chum BC6-1, which is highly resistant to sethoxydim, into maize (*Zea mays* L.) drived by a ubiquitin promoter. The substantial results indicated that the sethoxydim resistance of transgenic plants is increased, with a damage index of 33%, much lower than that of 97% in the untransformed plants. Additionally, the oil content of transgenic maize seeds was 24 to 65% higher than that of untransformed seeds. These results indicate that the ACCase transgenic plants can be used to develop new maize hybrids that are tolerant to herbicide CHDs with high oil content.

Key words: *Zea mays*, Acetyl-coenzyme A carboxylase (ACCase), oil content, transgenic plants, *Agrobacterium tumefaciens*

INTRODUCTION

Acetyl-coenzyme A carboxylase (ACCase) was first discovered nearly 50 years ago and has been studied extensively over the years (Wakil et al., 1983). Two forms of ACCase exist in plants. One is located in the plastids, the primary sites of plant fatty acid synthesis. This form is a “prokaryotic-type” multi-subunit enzyme and the other form, usually located in the cytoplasm, is a “eukaryotic-type” multifunctional enzyme. In the grass family (including wheat, rice and maize), however, the “eukaryotic-type” is located in both the cytosol and plastids “(Alban et al., 1994; Konishi and Sasaki, 1994; Gornicki et al., 1997; Zhao et al., 2004; Tong L, 2005).

Plant ACCases have attracted particular attention because the grass plastid ACCase is the active site of three chemically distinct classes of herbicides, aryloxyphenoxypropionates (APPs, also known as FOPs), cyclohexanediones (CHDs, also known as DIMs) and phenylpyrazolin. These classes of compounds reduce ACCase activity in meristematic tissue and kill sensitive plants by inhibiting their fatty acid biosynthesis. It has been reported that, grass ACCase in plastids is sensitive to these herbicides, but dicot and non-graminaceous monocot ACCases exhibit less sensitivity (Herbert et al., 1997; Christoffers et al., 2002). When these herbicides are used to kill weed grasses, they also kill the crop grasses, thus, greatly limiting their application. An alternative approach would be to produce grass crops highly tolerant to these herbicides.

There are some reports indicating that the sensitive form of the enzyme has an Ile residue, while the resistant form has a Leu residue at the putative herbicide-binding

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Abbreviations: ACCase, Acetyl-CoA carboxylase; **CHDs,** cyclohexanediones; **APPs,** aryloxyphenoxy propionates; **HR,** high resistance; **HS,** high sensitivity.

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site (Zagnitko et al., 2001). Additionally, a single Ile to Leu replacement at an equivalent position changes the wheat plastid ACCase from sensitive to resistant (Zagnitko et al., 2001). Furthermore, the cDNA (*foxACC-R* gene) of the foxtail millet (*Setaria italica*) line Chum BC6-1, which has been reported to show high resistance to sethoxydim, has the same Leu reside in this position compared with an Ile residue in sensitive lines (Wang T et al., 1997; Zhao et al., 2004).

In this study, we transformed the *foxACC-R* gene into the maize inbred line *Zong 3* using *Agrobacterium tumefaciens* mediated transformation (Zhang et al., 2001). Southern blot assay, RT-PCR detection and the test of sethoxydim tolerance on transgenic plants indicated that the *foxACC-R* gene was stably integrated into the maize genome; also, all these plants with the higher oil contents. Our objective was to develop maize lines that were highly resistant to sethoxydim; therefore, these transgenic lines can be incorporated into maize breeding programs in the future.

MATERIALS AND METHODS

Plasmid construction

The binary vector pCAMBIA3301 (Center for the Application of Molecular Biology to International Agriculture, Canberra, ACT, Australia) was used as a backbone for the construction of a plant expression vector. The bar gene, 35S promoter and GUS reporter gene were replaced by the *foxACC-R* (AY219175) driven by the ubiquitin promoter from maize to construct a marker-free p3301-ACCase vector (Figure 1).

Maize transformation

The approach used to generate transgenic maize plants was adapted from that described by Yang et al., 2001 and Zhang et al., 2001 The size of immature embryos are 1.0 to 2.0 mm, embryos were isolated from the maize inbred line *Zong 3* and put into liquid infection medium (D basal medium (Duncan et al.,1985), 68.5 gl^{-1} Suc, 36.0 gl^{-1} Glc, 100 µM AS (Sigma), pH 5.2) in 2-ml Eppendorf tubes (20 to 100 embryos per tube). Then, they were washed twice with this medium. The final wash was removed and 1.5 ml of *A. tumefaciens* suspension was added. The tubes were gently inverted 20 times and then, rested upright for five minutes with the embryos submerged. After infection, the embryos were transferred to the surface of co-cultivation medium (D basal medium, 20 gl^{-1} Suc, 10 gl^{-1} Glc, 0.85 mgl^{-1} silver nitrate, 100 µM AS, 8 gl^{-1} agar, pH 5.8). The embryos were incubated in the dark for three days at 25℃, after which they were transferred to resting medium (D basal medium, 20 gl^{-1} Suc, 10 gl^{-1} Glc, 0.85 mgl^{-1} silver nitrate, 250 mgl^{-1} cefotaxime, 8 gl^{-1} agar, pH 5.8) for seven days. Then, the embryos were moved to selection medium which was identical to resting medium with the addition of 5 µM sethoxydim and maintained for two weeks in the dark at 25℃. The concentration of sethoxydim was then increased to 10 µM for another three successive selections. Sethoxydim-resistant calli were placed on MS medium for regeneration. The shoots of 2 to 3 cm in height were moved to 1/2 MS rooting medium (Murashige and Skoog, 1962). Calli for regeneration and regenerated plantlets were grown in a growth chamber at 28℃ under fluorescent white light in 16:8 h light: dark cycle.

PCR analysis of transgenic maize plants

Genomic DNA was isolated from leaves of transgenic maize and untransformed plants by the SDS method for PCR analysis (Sabelli and Shewry, 1995). Primers for the ACCase gene (ACCase-S 5'-CAATGTGTATGCTACATGTTTTTGT-3' and ACCase-A 5'-ACCCCTGCAAAACCAGA-3') were used for PCR analysis, it is specific for foxtail millet and there was no corresponding band in the maize genome (Zhao et al., 2004). The PCR products were subjected to 0.8% agarose gel electrophoresis and stained by EtBr to visualize the expected band.

Southern blotting

Southern blot analysis was carried out by standard procedures as described by Sambrook and Russell (2001). DNA samples isolated from maize leaves of transgenic and untransformed plants were purified, concentrated and quantified. About 50 µg of genomic DNA was digested with 40 U of *Eco*RI (Promega) at 37℃ overnight. The digested DNA was separated by electrophoresis on a 0.8% agarose gel and blotted on Hybond- N+ nylon membrane (Amersham Pharmacia) in 0.4 mol/l NaOH. The probe (ACCase-S 5'-CAATGTGTATGCTACATGTTTTTGT-3' and ACCase-A 5'-ACCCCTGCAAAACCAGA-3') was random -primed labeled with (α-32P) dCTP. Southern hybridization was carried out overnight at 42℃. The hybridized filters were washed in 2 × SSC with 0.5% SDS at 42℃ for 30 min, twice in 1×SSC with 0.1% SDS at 65℃ for 30 min and twice in 2×SSC at 65℃ for 10 min then exposed to x-ray film (Fuji) at -70℃.

Semi-quantitative RT-PCR analysis of transgenic maize plants

Total RNA of the transgenic and untransformed plants was extracted according to the Trizol reagent (Invitrogen) protocol. All preparations of total RNA were evaluated by denatured agarose-glyoxal gel electrophoresis. First-strand cDNAs were synthesized using ImProm-II TM reverse transcriptase (Promega) and the ACCase transcript was amplified using foxtail millet ACCase gene primers (ACCase-S 5'- CAATGTGTATGCTACATGTTTTTGT-3' and ACCase-A 5'- ACCCCTGCAAAACCAGA-3'). At the same time, maize tubulin cDNA (Tubulin-S 5'-GCTATCCTGTGATCTGCCCTGA-3' and Tubulin-A 5'-CGCCAAACTTAATAACCCAGTA-3') was also amplified as a control.

Determination of oil content in transgenic maize seeds

For the oil content measurements, the transgenic and untransformed plants were grown in the same environment and harvested at the same time. Using a near infrared reflectance spectroscopy (NIRS) instrument (BRUKER, VECTOR22/N), 20 intact seeds in a revolving sample with a diameter of 50 mm were scanned 64 times at a wavelength region of 80 to 240 nm to calculate the average spectrum. In order to eliminate the influence of seed size and seed uniformity on the spectrum, each sample was replicated three times. Means for transgenic and untransformed plants were compared by using the two-sample t-test at the 5% confidence level as detected by LSD tests. The T_0 plants of PCR positive were self-pollinated to produce T_1 seeds, and T_1, T_2 plants that were highly resistant to sethoxydim were self-pollinated to produce T_3 seeds.

Sethoxydim spraying test

On a sunny day (>25℃), six-leaf stage T_1 plants were painted with

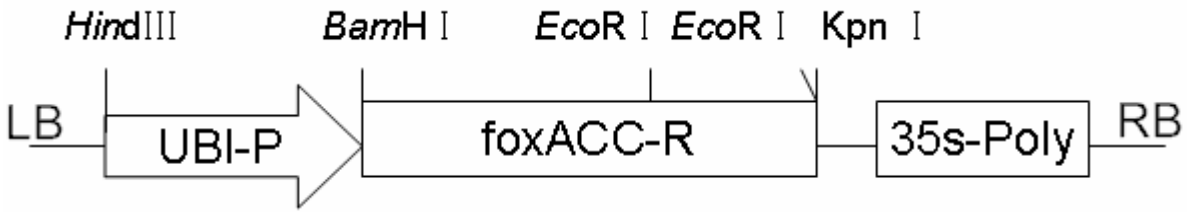

Figure 1. Structure of the expression vector p3301-ACCase. The ubiqutin promoter was fused with the foxACC-R gene. Horizontal lines represent short pieces of polylinker DNA that connects the gene elements. The plasmid vector pCAMBIA3301 was as the backbone, and for the future application, the bar gene, the 35S promoter and GUS reporter gene were deleted. Relevant restriction endonuclease sites are indicated.

a 625 mg/l solution of sethoxydim (12.5%; Nisso, Japan). Four- to six-leaf stage T_2 plants were sprayed in the field with a concentration of 1250 mg/l sethoxydim. The reaction of the plants to sethoxydim was recorded one week later. The degree of herbicide damage was evaluated according to the following criteria: level 0, no obvious chemical damage symptoms; level I, leaves show temporary chemical damage spots or slight growth suppression; level II, leaves show heavy chemical damage spots,loss of green coloration, shrinkage, abnormal growth or obvious growth suppression, but normal growth may be restored; level III, the growing point dies or continued growth is seriously suppressed; level IV, partial or the complete plant death.

The damage index was calculated according the following formula: damage degree index = (∑(damage level * number)/ total number * highest level) * 100% (Jiang, 1987). The resistance of maize to the herbicide was divided into five grades according to the damage degree index: HR (highly resistant) (index≤20); R (resistant) (20<index≤35); MR (moderate resistant) (35<index≤50); S (sensitive) (50<the index≤80); HS (highly sensitive) (index>80).

Phylogenetic analysis

Multiple sequence alignment was performed using ClustalX1.83 (Thompson et al., 1997) and manually adjusted using Jalview 2.07 (Clamp et al., 2004). A phylogenetic tree was constructed using the neighbor joining method in MEGA 3.1 (Kumar et al., 2004). The reliability of different phylogenetic groupings was evaluated using the bootstrap test (1000 bootstrap replications) implemented in MEGA 3.1.

RESULTS

Characterization of ACCase genes

The full-length cDNAs of plastid ACCase were cloned from foxtail millet Chum BC6-1 and wild type. Further sequence analysis showed that there is an Ile to Leu residue substitution at amino position 1780 (Zhao et al., 2004) in our laboratory, Foxtail millet Chum BC6-1 has a Leu residue at this position, while wild type has an Ile residue. Zagnitko et al. (2001) have reported that, a single-site Ile-to-Leu mutation in the ACC enzymes of many grasses was sufficient to confer resistance to both APPs and CHDs. Several years passed, some new species and new sequences appeared, so we conducted a sequence analysis again to determine whether this is universal or occasional (Figure 2; Table 1). As predicted, most grasses that were sensitive to APPs and CHDs have an Ile residue at this site in their plastid eukaryotic ACCases while all dicots and non-graminaceous monocots, which were resistant to the herbicides, have Leu at this position in their prokaryotic ACCases and in their cytosolic, eukaryotic ACCases. Grass cytosolic ACCase and animal and yeast ACCases, which are eukaryotic and are not sensitive to these herbicides, have Leu in this position.

Production of transgenic maize plants

Only those sethoxydim-resistant calli with white growing point were selected for successive rounds of selection (Figure 3a, b). Once shoots reached 2 to 3 cm in height, plantlets were moved to 1/2 MS rooting medium (Figure 3c) in bottles and then regenerated plantlets were grown in a growth chamber (Figure 3d). A total of 67 sethoxydim-resistant plantlets were regenerated and further screened by PCR analysis. Ten plantlets were PCR positive and could be self-pollinated to produce seeds. The PCR analysis was also performed on the progeny of each selected line (Figure 4). The results showed that each transgenic plant had the 195-bp band characteristic of foxtail millet ACCase cDNA, while untransformed plants lacked the corresponding band.

Integration and expression analysis of transgenic maize plants

*EcoR*I-digested leaf genomic DNAs from 10 PCR positive lines and an untransformed control were used for southern blot analysis and six lines (6, 17, 40, 46, 59 and 76) showed a 2.3 kb distinctive hybridization band, indicating the integration of the foxACC-R gene (Figure 5).

In addition, RT-PCR analysis was conducted on RNA from maize leaves. Each transgenic plant showed a corresponding band, which indicated the expression of the foxACC-R gene. However, untransformed plants lacked a detectable ACCase band (Figure 6).

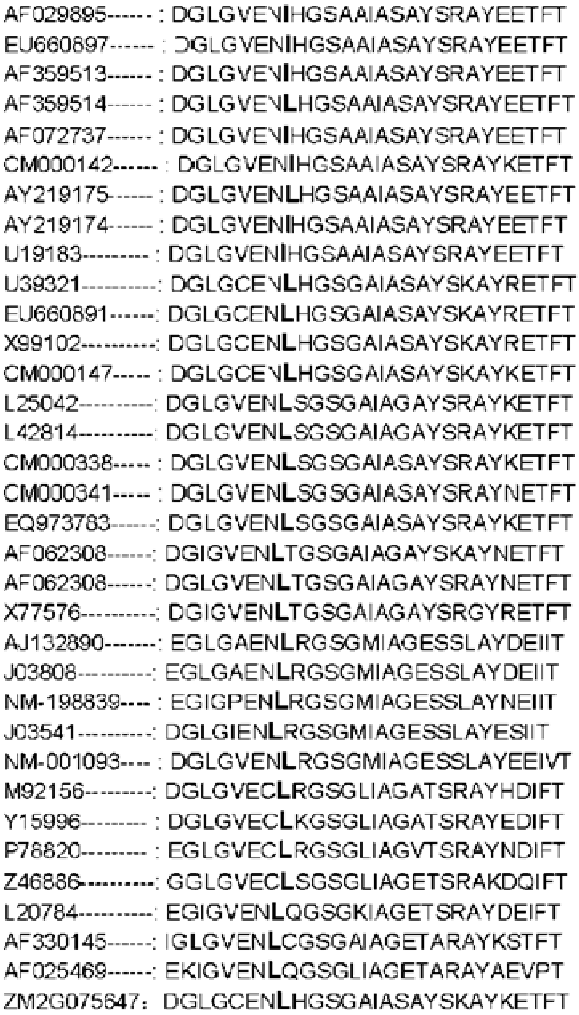

Figure 2. Alignment of the amino acid sequence of the CT domain of ACCase genes. The isoleucine /leucine is indicted in bold. ACCase sequences were obtained from GeneBank, left is the accession no. The species is listed in Table 1.

Comparison of oil content of transgenic and wild-type plants

The oil content of seeds was not determined for T_0 plants of PCR positive lines due to the potential epigenetic variation generated during plant tissue culture. Starting from T_1 plants, oil content was measured and compared with control plants. From the results of the comparison of T_1, T_2 and T_3 generation plants (Table 2; Figure 7), all positive plants of each line had significantly higher oil content than controls. Lines 40 and 46 had 54 to 65% higher oil content than the controls in the T_3 generation.

Sethoxydim tolerance assay of transgenic plants

To determine the response of the transgenic plants to sethoxydim, the fifth leaf of seedlings at the six-leaf stage was painted with sethoxydim at a concentration of 625 mg/l. Seven days after treatment, the transgenic seedlings showed normal growth (Figure 8a), while the untransformed plants wilted, turned brown and died in two weeks (Figure 8b).

T_2 plants were sprayed with 1250 mg/l sethoxydim (the commercial concentration) in the field. Seven days later, the transgenic plants all showed normal growth, while the untransformed plants wilted (Figure 8c, d and e). The degree of herbicide damage was evaluated for every plant of the T_2 generation (Table 3). The damage index of lines 40, 46 and 59 was lower than 20% and that of lines6, 17 and 76 were between 20 and 33% and the control had a damage index of 97%.

These results showed that the *foxACC-R* transgenic plants were more resistant than the control.

Table 1. The species chosen for the analysis and their accession number.

Accession number	Species name	Special residue
AF025469	*Caenorhabditis elegans*	L
U39321	*Triticum aestivum* (bread wheat)-Acc2	L
AF029895	*Triticum aestivum* (bread wheat)-Acc1	**I**
U19183	*Zea mays*	**I**
AY219175	*Setaria italica* (foxtail millet)-R	L
AY219174	*Setaria italica* (foxtail millet)-S	**I**
AF330145	*Toxoplasma gondii*	L
AF072737	*Avena sativa* (oat)	**I**
AF359513	*Lolium rigidum*-S	**I**
AF359514	*Lolium rigidum-R*	L
AJ132890	*Bos taurus* (cattle)	L
NM-198839	*Homo sapiens* (human)-ACC1	L
NM001093	*Homo sapiens* (human)-ACC2	L
J03541	*Gallus gallus* (chicken)	L
J03808	*Rattus norvegicus (*Norway rat)	L
L20784	*Cyclotella cryptica*	L
L25042	*Medicago sativa*	L
L42814	*Glycine max* (soybean)	L
M92156	*Saccharomyces cerevisiae*	L
P78820	*Schizosaccharomyces pombe*	L
X77576	*Brassica napus (rape)*	L
X99102	*Hordeum vulgare* subsp. *vulgare*	L
Y15996	*Emericella nidulans*	L
Z46886	*Ustilago maydis*	L
CM000142	*Oryza sativa* Japonica	**I**
EU660897	*Aegilops tauschii*	**I**
EU660891	*Aegilops tauschii*	L
CM000147	*Oryza sativa* Japonica	L
EQ973783	*Ricinus communis* (castor bean)	L
AF062308	*Arabidopsis thaliana*-ACC1	L
AF062308	*Arabidopsis thaliana*-ACC2	L
CM000338	*Populus trichocarpa*	L
CM000341	*Populus trichocarpa*	L

DISCUSSION

In plants, ACCase is a proven target for herbicide action. Genetic analyses have identified several single mutation sites in the CT domain that determine the herbicide resistance (Nikolau et al., 2003). A single-site Ile-to-Leu mutation at the position equivalent to residue 1705 in yeast ACC which represents an extremely subtle change in the side chain of the amino acid, is sufficient to confer resistance to both APPs and CHDs in the ACC enzyme of many grasses, while those that are resistant to these herbicides have a Leu residue at this position (Zagnitko et al., 2001; Delye et al., 2002). The second single-site mutation that confers resistance to herbicides is an Ile-to-Asn change (equivalent to residue 1967 in yeast ACC), again in the CT domain of ACC (Delye et al. 2003, 2004). Mutations at three additional sites in the CT domain have been identified from resistant weeds (Delye et al., 2005). An Asp/Gly change (equivalent to residue 2004 in yeast ACC) confers resistance to both APPs and CHDs, whereas a Trp/Cys and a Gly/Ala change (1953 and 2022 in yeast ACC, respectively) confer resistance to APPs only. From our results and analysis, we concluded that the change from Leu to Ile at residue 1705 is critical for the interaction of ACCase with CHDs. Interestingly, our phylogenetic analysis (Figure 9) of ACCase genes of

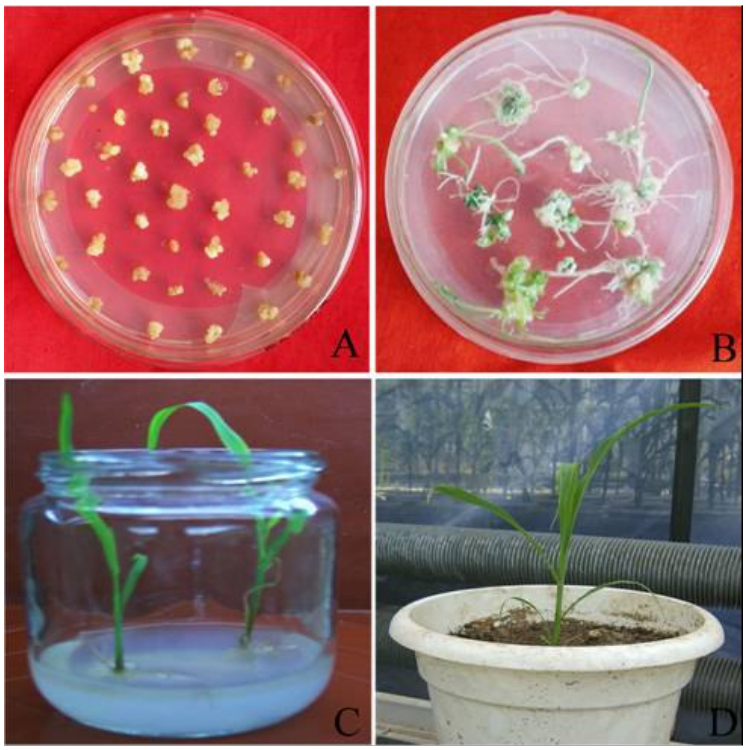

Figure 3. Production of transgenic maize plants. (A) Sethoxydim-resistant calli with the white growing point; (B) plantlet regeneration from the sethoxydim-resistant calli; (C) plantlets in 1/2 MS rooting medium; (D) regenerated plantlets in a growth chamber.

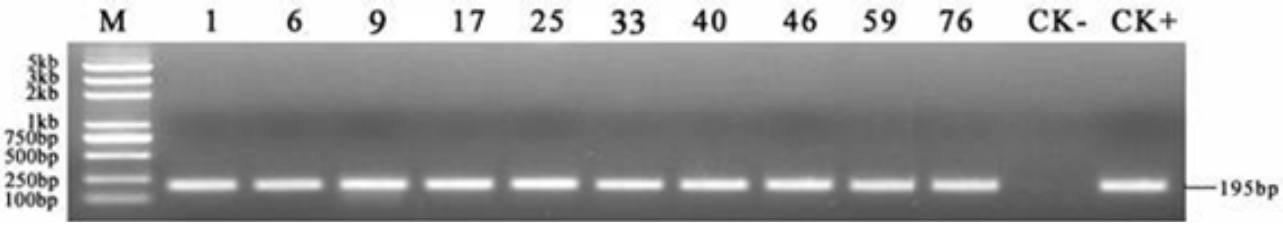

Figure 4. PCR detection of ACCase band (195 bp) in the posterity. M: DNA marker; lane 1, 6, 9, 17, 25, 33,40, 46, 59 and 76 represents the transgenic lines; lane CK- represent the untransformed plant *Zong3*; lane CK+ represent the plasmid p3301-ACCase.

Table 2. The oil contents of T_1, T_2 and T_3 generation plants.

Line	Oil content of T_1 generation (%)	Average oil content of T_2 generation (%)	Average oil content of T3 generation (%)
6	3.56±0.05^{de}	3.61±0.08^{c}	4.29±0.21^{c}
17	3.86±0.13^{cd}	4.30±0.25^{b}	4.47±0.35^{bc}
40	5.03±0.43^{b}	5.49±0.36^{a}	5.34±0.64^{a}
46	5.62±0.30^{a}	5.98±0.44^{a}	5.71±0.20^{a}
59	4.10±0.29^{c}	5.52±0.32^{a}	5.07±0.31^{ab}
76	5.09±0.18^{b}	4.54±0.39^{b}	4.58±0.43^{bc}
Z3	3.39±0.19^{e}	3.51±0.37^{c}	3.47±0.21^{d}

The oil contents of T2 and T3 generation are the mean value of four to five plants derived from one T1 plant. The number 6, 17, 40, 46, 59 and 76 represented independent transgenic lines, Z3 is untransformed inbred line. Means with standard errors were calculated from three replicates. Different letters represent significant difference at 5% confidence level as detected by LSD tests

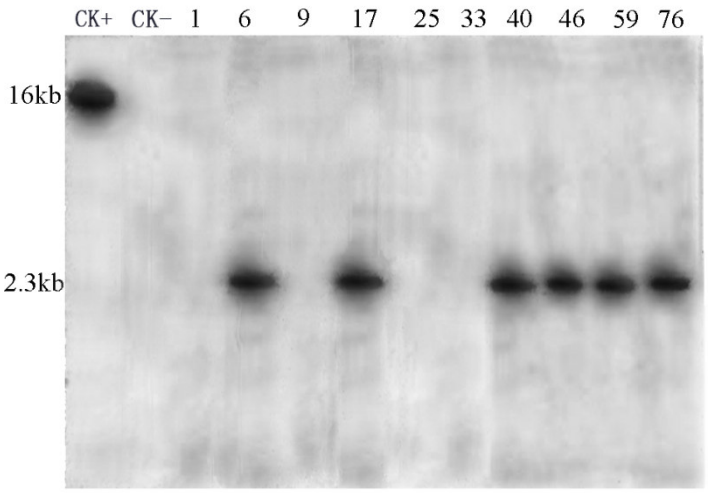

Figure 5. Southern blot analysis of T_2 transgenic maize plants. Aliquots of 50 µg of EcoRI -digested total maize leaf DNA was fractionated on 0.8% agarose gel, blotted to nylon membrane and probed with ^{32}P-labeled 195bp ACCase gene fragment. CK+ is the plasmid p3301-ACCase (16.37 kb) and CK- is the untransformed plant.

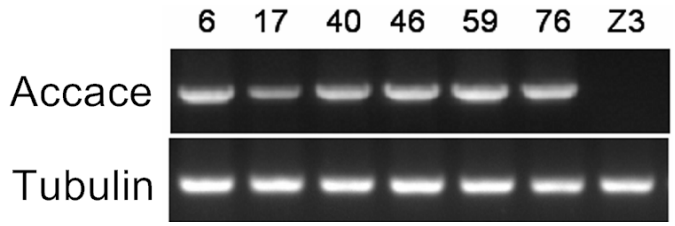

Figure 6. Detection of levels of the foxtail millet ACCase transcripts by semi-quantitative RT-PCR. Upper bands are the PCR fragments specific for Accase. Lower bands are the maize actin PCR fragments used as the control. 6, 17, 40, 46, 59 and 76 are the transgenic lines, Z3 is the untransformed plant.

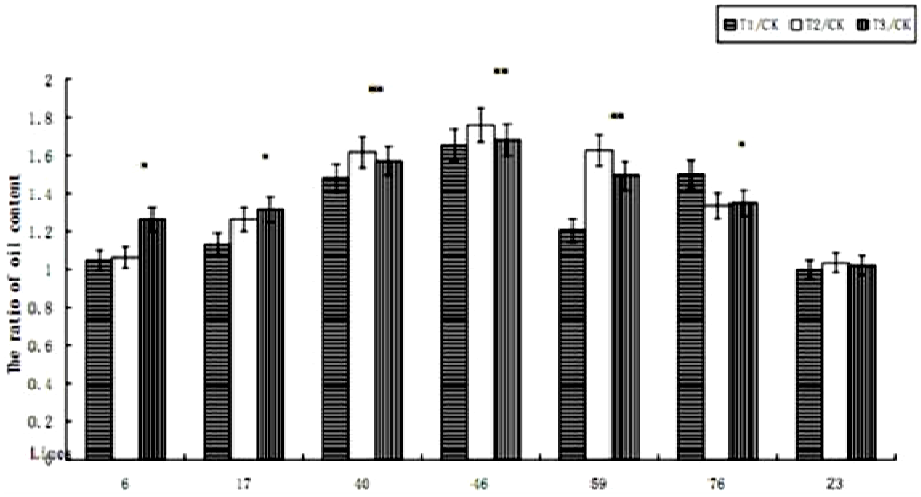

Figure 7. The ratio of oil content of the T_1, T_2 and T_3 generation plants. The data bars indicate the ratio of oil content of transgenic lines to untransformed control.

Table 3. The damage index of T_3 generation transgenic plants by herbicide.

Parameter	Transgenic line						Control
Line	6	17	40	46	59	76	Z3
The damage index (%)	30.00	22.22	16.67	14.28	13.33	32.14	96.67

The damage index is the mean value of fourteen to fifteen plants.

Figure 8. The sethoxydim resistance evaluation in the field. The fifth leaf of seedlings with 4 to 6 true leaves was pained by 625 mg/l of sethoxydim. Seven days after treatment, the WT plants turned wilt and brown (B), while the transgenic seedlings grown normal (A). The T_2 plants were sprayed with 1250 mg/l of sethoxydim in the field, (C) is the transgenic plant, (D) is the untransformed control. (E) Left line is the transgenic plants and right line is untransformed plants.

these eukaryotic and prokaryotic types indicates that, all types that have an Ile residue at this position are grouped into one subfamily, except for AF359514 and AY219175, which show a leu/Ile substitution at this position. These results suggest that the CT domain of ACCases in this subfamily has a special structure that can bind the herbicide and that this particular mutation may interfere with herbicide binding, leading to herbicide resistance. However, the exact mechanism is not clear.ACCase has been identified as an important regulatory enzyme for plant fatty acid synthesis by three *in vivo* approaches. Analysis of substrate and product pool sizes has implicated ACCase in the light/dark regulation of fatty acid synthesis in spinach leaves (Post-Beittenmiller et al.,1992) and chloroplasts (Post-Beittenmiller et al., 1991). The total oil content of seeds is increased approximately 5% by the expression of *Arabidopsis* ACCase in rapeseed plastids (Keith et al., 1997). In this study, over-expression of the plastid *foxACC-R* gene from a foxtail millet increased oil content from 24 to 65% in the T_3 generation over that of the control maize inbred line *Zong3* (Table 2). This result is consistent with that found in tobacco suspension cells, where ACCase is the apparent site of feedback inhibition of fatty acid synthesis when supplemented with exogenous fatty acids (Shintani and Ohlrogge, 1995).

Sethoxydim is a post-emergence herbicide used to eliminate weeds in dicotyledonous crops. It is absorbed by the leaves and transported throughout the phloem towards the apical meristem. Systemic herbicides require from several days to a few weeks to move throughout the vascular system of a treated plant. In monocots, necrotic zones develop in newly formed leaves, resulting in growth inhibition and cell death in one or two weeks. In dicots, effects of post-emergence herbicides have been reported under field conditions (Griffin and Habetz, 1989; Hart and Roskamp, 1998), but plants usually recover from injury, depending on growth conditions and season (Kapusta et al., 1986).

Our results indicate that the full-length cDNA of *foxACC-R*, when introduced into maize, leads to high resistance to the herbicide sethoxydim; the damage indices

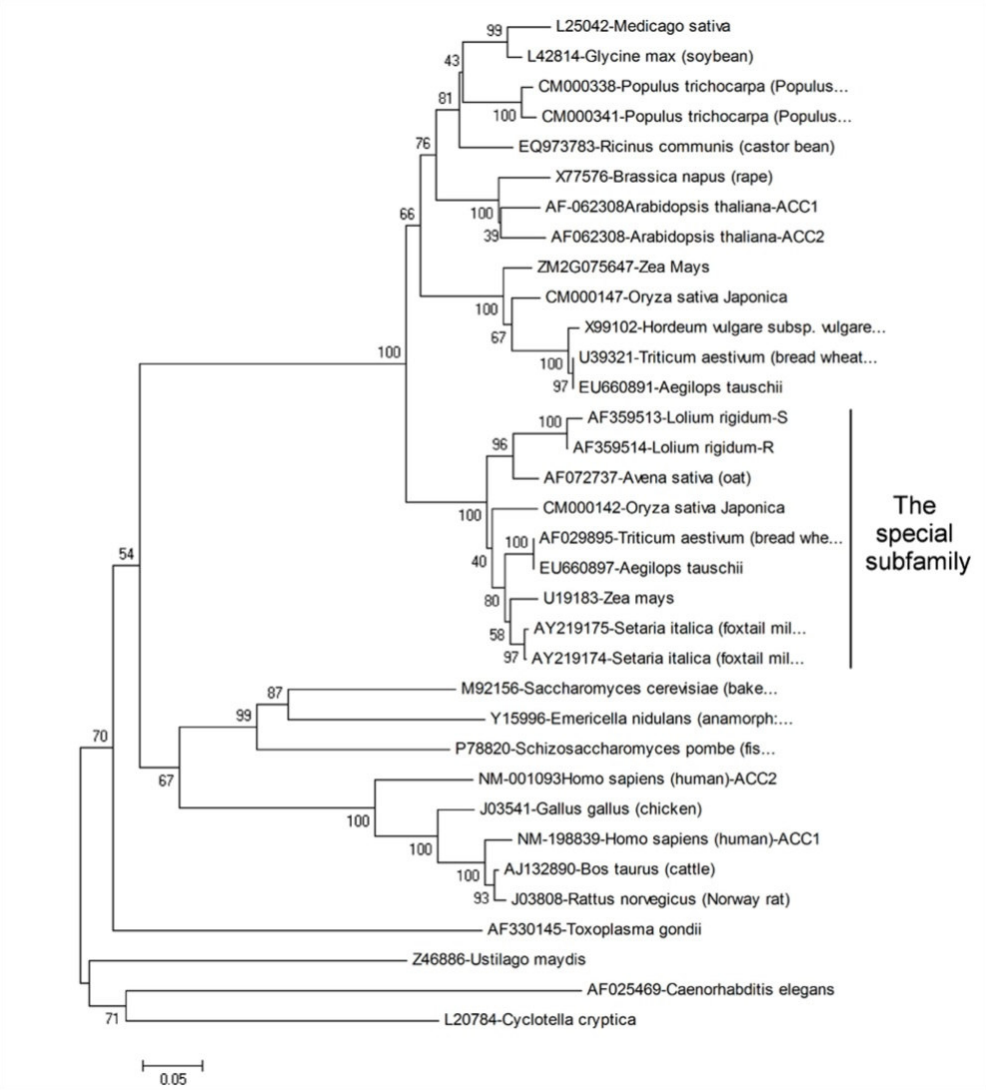

Figure 9. Phylogenetic analysis of the ACCase genes. The amino acid sequences of the ACCase gene were aligned by ClustalX1.83 and the phylogenetic tree was constructed using the NJ method in MEGA 3.1. All the types which have Ile residue were grouped into one subfamily, except for the AF359514 and AY219175 which is leucine /isoleucine substitution in this position.

of all transgenic lines were lower than 33%, while that of the control was 97%.

In summary, over-expression of the full-length cDNA of the *foxACC-R* gene in maize leads to increased resistance to sethoxydim. These lines might be used to develop new maize hybrids with herbicide tolerance in the near future.

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