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Transcriptional profiles of emasculated flowers of black locust (*Robinia pseudoacacia*) determined using the cDNA-AFLP technique

J.X. Wang[1]*, P. Sun[2]*, C.Q. Yuan[1], L. Dai[1], Y. Zhang[1], B. Wu[1], C. Long[1], Y.H. Sun[1] and Y. Li[1]

[1]National Engineering Laboratory for Tree Breeding,
Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants,
Ministry of Education, College of Biological Sciences and Technology,
Beijing Forestry University, Beijing, China
[2]Non-Timber Forestry Research and Development Center,
Chinese Academy of Forestry, Zhengzhou, China

*These authors contributed equally to this study.
Corresponding authors: Y.H. Sun / Y. Li
E-mail: sunyuhan@bjfu.edu.cn / yunli@bjfu.edu.cn

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ABSTRACT. Black locust (*Robinia pseudoacacia*) is a tree in the subfamily Faboideae, native to North America, that has been naturalized and widely planted in temperate Europe and Asia. Black locust has important ecological and economic value, but its quality needs improvement. Hybridization programs are important for black locust breeding, but the low rate of fruit set after controlled pollination limits both its breeding and that of other monoclinous plant species that share this problem. In this study, we investigated gene expression in emasculated black locust flowers using the cDNA-amplified fragment length polymorphism technique to determine why the rate of fruit set is low after controlled pollination. Flowers that were emasculated after being frozen in liquid nitrogen were used as controls. Changes in the flower transcriptome were more dramatic at 5 h after emasculation than at 48 h. Injury caused by emasculation decreased the expression levels of genes associated with metabolism, growth regulation, signal transduction, and photosynthesis, and it increased the

expression of genes related to stress-response metabolism, signal transduction, and promotion of senescence. The changes in the expression levels of these genes had negative effects on sugar metabolism, protein metabolism, lipid metabolism, energy metabolism, matter transport, signal transduction, osmotic regulation, pH regulation, and photosynthesis. Thus, emasculation accelerated flower senescence, resulting in low fruit set.

Key words: Black locust; *Robinia pseudoacacia*; cDNA-AFLP; Emasculation

INTRODUCTION

Black locust (*Robinia pseudoacacia*) is a monoclinous plant in the subfamily Faboideae that is native to North America, and it is widely planted and naturalized elsewhere in temperate Europe and Asia. Black locust has considerable ecological and economic value (Keeler, 1900; Yuan et al., 2013), but there are many problems that restrict cultivation of the tree. The breeding method most commonly used for black locust depends mainly on genotypic introduction and selection, and it has made significant contributions to breeding programs. However, the availability of desirable traits for introduction is limited, and genotypic selection is an uncertain basis for breeding programs. Space flight mutation-breeding technology has been applied to black locust. However, the main problems of this method are that the beneficial mutation frequency is still low, and the direction and character of the variation is difficult to control (Yuan et al., 2010). Breeding programs based on controlled pollination provide an important alternative approach for breeding black locust. However, low fruit set rates after controlled pollination result in insufficient numbers of offspring for progeny selection, and most flowers that result from artificial hybridization abscise approximately 1 week after artificial pollination. The low rate of fruit set associated with the use of artificial hybridization is shared with other monoclinous plant species such as sweet cherry (Hedhly et al., 2009), plum (Guerra et al., 2010), amarelle, and other plants with hermaphroditic flowers (Guerra et al., 2010). This phenomenon has presented a major impediment to the artificial cross breeding of black locust and other monoclinous plants.

The cDNA-amplified fragment length polymorphism (AFLP) technique is an efficient and simple mRNA fingerprinting method for the isolation of differentially expressed genes (Bachem, 1996). This technique is based on the selective PCR amplification of restriction fragments produced from a restriction digest of total genomic DNA (Vos et al., 1995). This is a robust and high-throughput tool for analyzing genome-wide expression and for gene discovery where prior knowledge of gene sequences is not a prerequisite. The high sensitivity of this method makes the identification of rare transcripts possible (Fukumura et al., 2003). The cDNA-AFLP technique has been improved to avoid the possibility of multiple-transcript-derived fragments (TDFs) arising from a single gene or cDNA (Breyne et al., 2003).

In this study, we used the cDNA-AFLP technique to investigate the effect of emasculation damage on gene expression to obtain a more comprehensive understanding of the effects of damage caused by emasculation on ageing and the rate of seed set after artificial hybridization.

MATERIAL AND METHODS

Plant material

Flowers were collected from an adult black locust tree exhibiting normal growth and

development at the Mijiabao Forestry Station in Beijing, Yanqing, China. Pollen was collected from ripe flowers, stored in glass bottles containing silica gel at 4°C, and used within 3 days after collection to avoid viability loss. To emasculate flowers, we removed partial petals and entire stamens 1 day prior to anthesis to avoid contamination from self-pollen, and the flowers were placed in paper bags (Figure 1). After removal from the bags at 24 h after emasculation, the emasculated flowers were pollinated artificially with prepared pollen, and the flowers were returned to bags for 7 days.

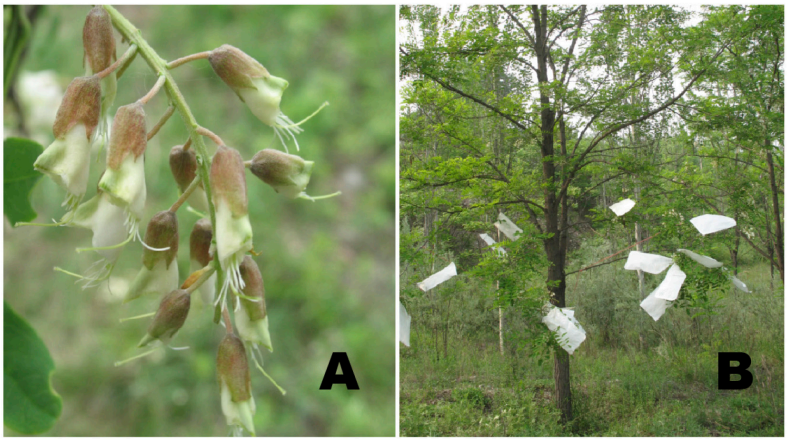

Figure 1. Treatment of black locust flowers by (**A**) emasculation and (**B**) bagging.

Unemasculated control flowers were placed in paper bags 1 day prior to anthesis, and were removed from the bags immediately before beginning artificial pollination. After pollination, the flowers were bagged for 7 days. Therefore, differences between control and emasculated flowers were due only to the effects of emasculation (Sun et al., 2013).

We collected the emasculated and control flowers at 5 and 48 h after emasculation. To ensure consistency in the processing of materials, the control flowers were first frozen in liquid nitrogen. The frozen corolla and stamens were treated identically to those of emasculated flowers so that differences between the treatment and control would be due only to physiological responses to emasculation and not to differences in sample processing. All samples were frozen immediately in liquid nitrogen and transported to the laboratory, where they were stored at -80°C until use for cDNA-AFLP analysis.

Total RNA extraction, mRNA isolation, and cDNA synthesis

Total RNA was extracted from 100-g tissue samples using an Aidlab EASYspin Plus Rapid Rlant RNA Extraction Kit (Aidlab Biotechnologies Co, Ltd., China). RNA quality and quantity were determined using a NanoDrop1000 spectrophotometer (NanoDrop Technologies, USA). RNA samples with A_{260}/A_{280} ratios between 1.9 and 2.1 and A_{260}/A_{230} ratios between 2.0 and 2.5 were assessed for RNA integrity by electrophoresis on 1.5% agarose gels alongside an RNA ladder (Invitrogen, Carlsbad, CA, USA). RNAs that met the quality criteria were used to synthesize cDNA. PolyA mRNA was isolated from total RNA (0.5-1.0 g) using a PolyATract mRNA isolation system (Promega, Madison, WI, USA), according to the manufacturer instructions. For cDNA synthesis, mRNAs from the collected tissues were pooled and used for first-strand cDNA synthesis, followed by second-strand synthesis, using a SuperScript double-stranded cDNA synthesis kit (Invitrogen) per the manufacture instructions with custom synthesized poly-d[T]25 oligonucleotides (Alpha DNA, Canada).

cDNA-AFLP analysis

Approximately 500 ng double-stranded cDNA were used for standard AFLP template production, according to the method of Vos et al. (1995). The cDNA was digested using *Mse*I and *Eco*RI restriction enzymes (New England Biolabs, Inc., USA). The digested products were ligated to adapters using the following primer sequences: Eup, 5'-CTCGTAGACTGCGTACC-3'; Edown, 5'-AATTGGTACGCAGTCTAC-3'; Mup, 5'-GACGATGAGTCCTGAG-3'; and Mdown, 5'-TACTCAGGACTCAT-3'.

Equal amounts of preamplified products were amplified using two primers with selective nucleotides at their 3'-ends: E0, 5'-GACTGCGTACCAATTCA-3' and M0, 5'-GATGAGTCCTGAGTAA C-3'. A total of 51 primer combinations were tested. Four microliters of the AFLP products were heat-denatured and resolved on a 6% denaturing polyacrylamide sequencing gel containing 0.5X TBE electrophoresis buffer using a Sequi-Gen system (Bio-Rad Laboratories, Hercules, CA, USA). The gels were silver-stained using a Silver Sequence kit (Promega) according to the manufacturer instructions. All reactions for restriction digestion, adapter ligation, preamplification, and selective amplification were performed according to procedures described by Subudhi (1998).

Gel bands corresponding to polymorphic fragments were excised from the gel with a scalpel after wetting the bands with 6 µL ddH_2O. The gel bands were placed in 20 µL ddH_2O, incubated at 95°C in a water bath for 10 min, and centrifuged at 12,000 rpm for 5 min, and the supernatants were recovered as templates. Reamplification of the fragments was performed using the same selective amplification primers used for preamplification. Reactions contained 8.5 µL ddH_2O, 2 µL E0 (selective amplification primer, 10 pM/µL) or M0 (selective amplification primer, 10 pM/µL) primers, 22.5 µL 2X *taq* Mix (TaKaRa, Dalian, China), and 10 µL enzyme ligation product. PCR conditions used in reamplification were the same as those used for the preamplification step.

Sequencing of fragments and gene ontology annotation

Reamplified PCR products (5 µL) were resolved on 2% agarose gels. Those that produced clear, single bands were submitted for sequencing (Biomed Corporation, China). The sequences were analyzed using the BLASTx software (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to identify homologous proteins in the GenBank database at the National Center for Biotechnology Information (NCBI). For sequences with low homology to protein sequences, the BLASTn software (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) was used to identify homologous gene fragments in the GenBank database at NCBI. The Uniprot Protein (UniProtKB, http://www.uniprot.org/) database was searched to further characterize the potential functions of the homologous proteins and gene fragments.

Verification of expression using real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed using a 7500 Fast Real-Time PCR machine (Applied Biosystems, CA, USA) and a SYBR Ex Taq Kit (TaKaRa). The PCR program included an initial denaturation step at 95°C for 3 min; 40 cycles of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, and a final melting curve of 70°-95°C. PCRs were prepared in 20-µL volumes containing 2 µL 10-fold-diluted synthesized cDNA, 10 µL 2X SYBR Premix Ex Taq, 0.4 µL 10 µM primer, 0.5 µL 50X ROX reference dye, and 6.9 µL ddH_2O. The melting curve was used to verify the specificity of amplified fragments. All reactions were

performed in triplicate for technical replication, with the biological replication of three plants per test. Expression levels were standardized using *ACTIN* gene expression, and the RT-qPCR data were analyzed using ANOVA. The primer pairs used for RT-qPCR are listed in Table 1.

Table 1. Primer pairs used for RT-PCR.

Gene name	Forward primer	Reverse primer	PCR efficiency (%)
T3	AAGAGCCATCGCAGGTAT	TTGGGATTGTTCAAGGGA	99.1
T5	CTTCTATCCTGGTTCTCA	GGAAGTTTCAGTTTCCTA	101.6
T12	GTAAGTGGCTGGGTTGCG	GAAGTGGGTGGGTTGGAG	99.3
T13	AAGGGCTCCAATTTCTC	TGAAGCCATTGGTTGAT	101.1
T18	GAGCATCATCAAAGGGAC	CAAGTGGGACATAAGTTCA	99.5
T25	ATTATTCCGTGTCTTTCG	GGTTACAGGGAGTGTTCTA	98.8
T90	ACAGGACAAACGGCATAA	GTCTTCAGAACTGGAGGG	102.4
T96	TGGGACTCTGGTGTAAGC	CTCCGATGGTAGATTTGG	102.3
Actin	TTGCCTTGGATTATGAACA	GATGGCTGGAACAGAACTT	99.2

RESULTS

Sequence analysis

We used 80 pairs of selective amplification primers to identify differences in gene expression between emasculated and control flowers at 5 and 48 h time points after emasculation. Approximately 3000 DNA fragments were amplified (Figure 2), 100 of which were TDFs. The sequenced products of reamplification included those that resolved as clear, single bands using agarose gel electrophoresis and those that exhibited band sizes that were the same on a 6% polyacrylamide gel. Seventy-three fragments were sequenced successfully. Both BLASTn and BLASTx softwares were used to identify homologous protein and gene sequences in the GenBank database, and functional identifications were refined by searching the UniProtKB database. The results of the homology analysis are presented in Table 2.

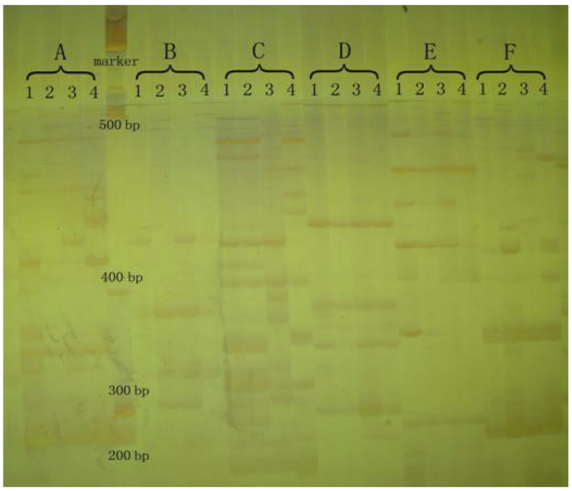

Figure 2. Electrophoretogram of cDNA-AFLP. A, B, C, D, E, F indicate different primer pairs. *Lane 1*: control flowers at 5 h after emasculation. *Lane 2*: flowers at 5 h after emasculation. *Lane 3*: control flowers at 48 h after emasculation. *Lane 4*: flowers 48 h after emasculation.

Table 2. Sequences with homology to transcript-derived fragments (TDFs) generated from black locust and their potential functions.

TDF	Length (bp)	Accession No.[a]	Homologous sequence	E-value[b]	Function
T3	245	XP_003607413.1	GRAS family transcription factor fro*m Medicago truncatula*	1e-35	Transcription factor
T5	430	AC150442.2	*Medicago truncatula* chromosome 7 clone mth2-2o12, complete sequence	1e-04 (BLASTn)	Unknown
T6	317	XP_003621281.1	Xenotropic and polytropic retrovirus receptor-like protein fro*m Medicago truncatula*	6e-17	Induced apoptosis
T7	421		No homology		
T9	315		No homology		
T11	381	XM_003531551.1	Predicted *Glycine max* seed linoleate 9S-lipoxygenase-2-like (LOC100816356), mRNA	3e-10 (BLASTn)	Damage stress response
T12	300		No homology		
T13	276	XM_003551615.1	*Glycine max* SKP1-like protein 21-like (LOC100782740), mRNA	2e-94 (BLASTn)	Can form a ubiquitin ligase that promotes proteolysis
T14	631	XM_003532472.1	*Glycine max* microtubule-associated protein 70-2-like (LOC100785173), mRNA	8e-08 (BLASTn)	Plant-specific microscopic binding proteins regulating organ growth directionality
T15	227	XM_003630465.1	*Medicago truncatula* tubulin beta chain (MTR_8g098360) mRNA, complete CDS	5e-11 (BLASTn)	Tubulin related to cytoskeleton
T17	527	EJJ19729.1	Hypothetical protein A176_3548 from *Chondromyces apiculatus DSM 436*	0.013	Unknown
T18	351	XP_003528576.1	Uncharacterized protein LOC100807956 from *Glycine max*	2e-26	Unknown
T19	470	ACI42832.1	NAC-like transcription factor from *Arachis hypogaea*	5e-19	Transcription factors associated with cell division
T22	922	XM_003521277.1	*Glycine max* myosin-Va-like (LOC100795648), mRNA	5e-13 (BLASTn)	Intercellular signaling
T23	344	XP_002532724.1	Putative inter-alpha-trypsin inhibitor heavy chain, from *Ricinus communis*	1e-10	Metabolism
T24	461	XP_003522035.1	Putative disease-resistance protein At4g11170-like from *Glycine max*	6e-18	Disease resistance
T25	590	XP_003522035.1	Putative disease-resistance protein At4g11170-like from *Glycine max*	1e-25	Disease resistance
T26			No homology		
T27			No homology		
T28	81	ZP_06980144.1	Autonomous glycyl radical cofactor from *Neisseria* sp *oral taxon 014 str. F0314*	0.008	Hypoxia stress reaction
T31	443	AES74028.1	Lateral signaling target protein-like protein from *Medicago truncatula*	7e-05	Growth factor receptor signal transduction inhibitors
T32			No homology		
T33			No homology		
T34	344	AP010316.1	*Lotus japonicus* genomic DNA, chromosome 4, clone: LjT49M15, TM2003, complete sequence	0.003	Unknown
T35			No homology		
T36			No homology		
T38			No homology		
T39	372	XP_001950328.1	AP-2 complex subunit mu-1-like from *Acyrthosiphon pisum*	4e-15	Transport proteins
T43	250	NP_194773.1	UDP-D-glucuronate 4-epimerase 1 from *Arabidopsis thaliana*	1e-07	Promotes cell wall synthesis
T44	292		No homology		
T45	238	NM_001255202.2	*Glycine max* E3 ubiquitin-protein ligase SDIR1-like (LOC100816448), mRNA	2e-04 (BLASTn)	Senescence-associated gene
T46	338	BAB33421.1	Putative senescence-associated protein from *Pisum sativu*m	0.001	Senescence
T47	337	XP_003540337.1	Predicted diacylglycerol kinase iota-like from *Glycine max*	0.87	Energy metabolism
T48	295	XP_003619839.1	D-tagatose-1,6-bisphosphate aldolase subunit gatY from *Medicago truncatula*	1e-10	Glycometabolism
T49	455	XP_003536202.1	Predicted 3-ketoacyl-CoA synthase 11-like from *Glycine max*	1e-13	Long chain fatty acid synthesis
T53	401	AB059562.1	*Aphanothece halophytica* psbV and apNhaP genes for cytochrome c550, Na^+/H^+ antiporter, partial CDS	0.59 (BLASTn)	Regulation of cellular pH
T57	253	GQ108962.1	Uncultured eukaryote clone nbw720b02c1 16S ribosomal RNA gene, partial sequence; plastid	3e-09 (BLASTn)	Associated with protein synthesis
T62	442	XM_003523497.1	Predicted *Glycine max* uncharacterized protein LOC100779033 (LOC100779033), mRNA	0.65 (BLASTn)	Unknown
T63	349	FJ617270.1	*Lotus japonicus* auxin response factor 3b (ARF3b) mRNA, complete CDS	0.041 (BLASTn)	Promotes gametophyte development
T64			No homology		
T65			No homology		
T66	296	EU835198.1	*Triticum turgidum* subsp *dicoccoides* clones BAC 391M13 and BAC 1144M20 genomic sequence	1.5 (BLASTn)	Antibacterial, antiviral
T67	293	EGZ18812.1	Hypothetical protein PHYSODRAFT_255429 from *Phytophthora sojae*	0.34	Unknown

Continued on next page

Table 2. Continued.

TDF	Length (bp)	Accession No.[a]	Homologous sequence	E-value[b]	Function
T68	237	NM_001255202.2	*Glycine max* E3 ubiquitin-protein ligase SDIR1-like (LOC100816448), mRNA	0.027 (BLASTn)	Suppress formation, promote the yellowing effect of dark environment
T69	339	FJ417059.1	*Myxidium bergense* isolate M0413 28S large subunit ribosomal RNA gene, partial sequence	0.003 (BLASTn)	Associated with protein synthesis
T70	381	AP009794.1	*Lotus japonicus* genomic DNA, clone: LjB14K02, BM1736b, complete sequence	0.013 (BLASTn)	Unknown
T72	290		No homology		
T73	545		No homology		
T76	348	XP_002532724.1	Putative inter-alpha-trypsin inhibitor heavy chain from *Ricinus communis*	5e-10	Metabolism
T77	318	XP_003621281.1	Xenotropic and polytropic retrovirus receptor-like protein from *Medicago truncatula*	4e-12	Induced cell death
T79	358	ZP_08475450.1	Hypothetical protein HMPREF9455_03616 from *Dysgonomonas gadei* ATCC BAA-286	0.31	Promotes membrane formation
T80	192	XP_003519895.1	Predicted low-quality protein: uncharacterized hydrolase yugF-like, partial from *Glycine max*	3e-13	Carboxylesterase activity, promotes metabolism
T86	176	AEI83223.1	Developmentally regulated GTP-binding protein from *Dimocarpus longan*	1e-22	Promotes cell proliferation, differentiation, and death
T87	161	XP_003622762.1	Auxin-induced protein in root cultures from *Medicago truncatula*	3e-07	Auxin-induced proteins
T88	277	XP_002884270.1	GHMP kinase family protein from *Arabidopsis lyrata* subsp *lyrata*	2e-26	Galactose kinase activity
T89	194	XP_003536098.1	Predicted uncharacterized protein LOC100814635 from *Glycine max*	7e-16	Unknown
T90	225	XP_003535891.1	Predicted low-quality protein: probable calcium-binding protein CML49-like from *Glycine max*	1e-16	Inhibition of cell division
T91	150	XP_003544634.1	Predicted uncharacterized protein LOC100788165 from *Glycine max*	3e-05	Unknown
T92	184	AP010402.1	*Lotus japonicus* genomic DNA, chromosome 5, clone: LjT02L21, TM1235b, complete sequence	2e-22 (BLASTn)	Unknown
T93	188	AP010402.1	*Lotus japonicus* genomic DNA, chromosome 5, clone: LjT02L21, TM1235b, complete sequence	2e-27 (BLASTn)	Unknown
T94	181	EAZ07296.1	Hypothetical protein OsI_29544 from *Oryza sativa indica* group	1.00	Aspartic acid in the peptide chain incision enzyme
T95	206	XP_002280427.1	Predicted 6,7-dimethyl-8-ribityllumazine synthase, chloroplastic-like from *Vitis vinifera*	6e-16	Promotes riboflavin formation
T96	249	XP_003537971.1	Predicted ras-related protein Rab11C-like from *Glycine max*	2e-17	Adjust osmotic pressure
T100	248	Z73951.1	*Lotus japonicus* mRNA for small GTP-binding protein, RAB11C	2e-61 (BLASTn)	Promotes nucleus and cytoplasm material exchange, chromosome condensation, and cell cycle regulation
T101	263	BAB10743.1	Retroelement polyprotein-like from *Arabidopsis thaliana*	1e-27	Reverse transcription factor, bind nucleic acid and zinc ions
T102	430	AC150442.2	*Medicago truncatula* chromosome 7 clone mth2-2o12, complete sequence	1e-04 (BLASTn)	Unknown
T103	317	XP_003621281.1	Xenotropic and polytropic retrovirus receptor-like protein from *Medicago truncatula*	5e-17	Induced cell death
T104	430	AC150442.2	*Medicago truncatula* chromosome 7 clone mth2-2o12, complete sequence	1e-04 (BLASTn)	Unknown
T105			No homology		
T106	421	BT143657.1	*Lotus japonicus* clone JCVI-FLLj-20E19 unknown mRNA	0.18 (BLASTn)	Unknown
T107			No homology		
T108	381	AJ276265.1	*Cicer arietinum* partial mRNA for lipoxygenase	2e-13 (BLASTn)	Promotes ageing, damage response associated with jasmonic acid synthesis
T109			No homology		

[a]GenBank accession No. of proteins and genes homologous to the TDFs generated in this study. [b]Homology scores derived from analyses using the BLASTn software are indicated by the annotation "(BLASTn)" below the score. All other homology scores were derived from analyses using the BLASTx software.

Expression analysis and functional classification of TDFs

The differential expression characteristics of the TDFs are summarized in Table 3. According to Bhalerao et al. (2003), we divided the TDFs into eight categories based on the functions of their homologous sequences in GenBank: cell metabolism, ageing-related (senescence), stress responses, signal transduction and growth regulation, transcription factor, photosynthesis, unknown function, and no homology. All of the categories containing TDFs are shown in Table 4.

TDFs related to cell metabolism

Of the sequenced TDFs, 19.2% had functions related to cell metabolism, indicating the marked effect of emasculation on cell metabolism. The expression levels of some genes with functions related to cell metabolism were reduced by emasculation. The TDF designated T48 was homologous to aldolase, which catalyzes the catabolism of galactitol, N-acetyl-galactosamine, and d-galactosamine, and it plays an important role in glucose metabolism (Brinkkoetter et al., 2002). T48 was expressed highly in control flowers at 5 and 48 h, but its expression was reduced in emasculated flowers at both time points.

The homolog of T49 maintains the length of very-long-chain fatty acids between 26 and 30 carbon atoms. Very-long-chain fatty acids are hydrophobic, high-molecular weight polymers that prevent plant leaves from becoming too dry, providing a suitable environment for pollen (Trenkamp et al., 2004). The T49 homolog also improves the performance of the cuticle of epithelial cells by catalyzing lipid synthesis (Pruitt et al., 2000). In addition, the T49 homolog prevents abnormal pollen hydration and germination, promotes ovule formation, and regulates epithelial cell growth during pistil morphogenesis (Lolle and Cheung, 1993). These results indicated that T49 is involved in pollen and ovule development. T49 was expressed at high levels only in the control flowers at 5 h, and its expression was barely detectable in emasculated flowers at 5 h. Thus, emasculation had a negative effect on the normal development of male and female reproductive organs.

The homolog of T79 promotes the insertion of intrinsic proteins into the cell membrane, and it plays a role in intrinsic protein folding and complex formation. It also promotes the synthesis and translocation of lipoprotein in the cell membrane, suggesting that T79 may play important roles in cell membrane function. T79 was highly expressed in the control flowers at 48 h, but showed little expression in the emasculated flowers at 48 h, which suggests that emasculation may affect cytomembrane function.

The homolog of T80 was a carboxyl esterase gene, the product of which catalyzes the hydrolysis of esters, sulfate esters, and amides. The homolog of T94 has endopeptidase activity, and is involved in protein metabolic processes (Yu et al., 2005). The homolog of T95 promotes the synthesis of riboflavin, which is involved in *in vivo* biological oxidation, energy metabolism, and metabolism associated with cell growth. T80, T94, and T95 were all expressed highly in the control flowers at 5 h, but their expression was almost undetectable in the emasculated flowers, indicating that emasculation had a negative impact on the material and energy metabolism of cells and on pollen and ovule growth. These effects may explain the premature senescence and abscission and the low fruit set rate of emasculated flowers. T57 was homologous to a 16S ribosomal ribonucleic acid (rRNA) gene, and it was expressed in the control flowers at 5 h; however, it showed little expression in the emasculated flowers.

Table 3. Expression characteristics of transcript-derived fragments (TDFs).

TDF	Control 5 h	Emasculation 5 h	Control 48 h	Emasculation 48 h
T3		●		●
T5	○	●		
T6	○	●	○	●
T7	○	●		
T9	○	●	○	●
T11		●		
T12	●	●		
T13	●	○	●	○
T14		●	○	○
T15		●		
T17		●		
T18		●		●
T19		●		●
T22		●		●
T23	○	●		
T24	○	●		
T25	○	●		
T26		●		
T27	●		●	
T28		●		
T31		●		
T32	●			
T33	●	○	●	○
T34	●			
T35		○		○
T36		○		
T38		●		
T39	●		●	
T43		○		
T44	●	○	●	
T45				●
T46		●		
T47		●		
T48	●	○	●	○
T49	●			
T53	●			
T57	○			
T62		●		●
T63	●			
T64		●		
T65		●		●
T66	○	●		
T67	●		●	
T68				○
T69	○	●		
T70			●	
T72	●	○	○	
T73	○	○	●	
T76	○	●		
T77		●		●
T79			●	
T80	●			
T86			●	
T87		●		
T88				●
T89				●
T90	●	●		
T91		●		
T92	○	○	○	●
T93				●
T94	●			
T95	●			
T96	●		●	
T100			●	
T101		●		
T102	○	●		
T103	○	●	○	●
T104	○	●		
T105	○	●		
T106	○	●	○	●
T107	○	●		
T108		●		
T109	●	●		

●cDNA band on a gel was distinct, and the gene expression level was high. ○cDNA band on a gel was indistinct, and the gene expression level was low. The absence of ● or ○ indicates that expression was not detected.

Table 4. Functional classification of differentially expressed genes induced by emasculation injury.

TDF function	TDF ID	TDF quantity	Percentage (%)
Cell metabolism	T15, T23, T43, T47, T48, T49, T57, T69, T76, T79, T80, T88, T94, T95	14	19.2
Ageing-related	T6, T13, T45, T46, T68, T77, T103	7	9.6
Stress response	T11, T24, T25, T28, T66, T108	6	8.2
Signal transduction and growth regulation	T14, T22, T31, T39, T63, T86, T87, T90, T96, T100	10	13.7
Transcription factor	T3, T19, T101	3	4.1
Photosynthesis	T53	1	1.4
Unknown function	T5, T17, T18, T34, T62, T67, T70, T89, T91, T92, T93, T102, T104, T106,	14	20.5
No homology	T7, T9, T12, T26, T27, T32, T33, T35, T36, T38, T44, T64, T65, T72, T73, T105, T107, T109	18	23.3
Total		73	100.0

TDF = transcript-derived fragments.

Emasculation promoted the expression of multiple genes related to cellular metabolism. T15 was homologous to tubulin, and it was expressed highly at 5 h after emasculation. The homolog to both T23 and T76 regulates the localization, synthesis, and decomposition of hyaluronan, which is involved in water retention, and it also plays important roles in the maintenance of normal biological cell function. Emasculation increased the expression of both T23 and T76. A T43 homolog catalyzes the synthesis of negatively charged monosaccharides, which are essential components of pectin (Usadel et al., 2004), and its expression also increased at 5 h after emasculation. A T47 homolog promotes the synthesis of ATP, consistent with a role in cellular metabolism, and it was highly expressed at 5 h after emasculation. T88 is homologous to galactokinase, which catalyzes the dephosphorylation of ATP, and its expression increased dramatically in flowers at 48 h after emasculation. These results indicated that responses to emasculation in flower organs include increased water retention, increased cell wall synthesis, and increased energy metabolism. These responses reduce the negative effects of injury from emasculation on flower development. T69 was homologous to a 28S large subunit rRNA gene involved in protein synthesis, and its expression increased at 5 h after emasculation relative to the control flowers.

TDFs related to senescence

TDFs related to senescence accounted for 9.6% of all sequenced TDFs. T6, T77, and T103, which promote Xpr1 protein expression, were homologous. Xpr1 induces apoptosis after binding to heterophil (Bamunusinghe et al., 2013) or polytropic retrovirus in humans. This result suggested that T6 induces senescence and apoptosis of black locust flower organs after retrovirus infection. T6, T77, and T103 expression in flowers was higher at 5 and 48 h after emasculation compared to the control flowers. T45 and T68 were both homologous to ubiquitin ligase, which promotes protein ubiquitination and subsequent hydrolysis into small polypeptide fragments and amino acids. T45 and T68 were homologous, and the expression of both increased in flowers at 48 h after emasculation. The function of a T46 homolog was related to the decomposition of metaprotein in chloroplasts, and it was expressed highly at 5 h after emasculation.

Because the *Arabidopsis*-SKP1-like (ASK) gene, homologous to T13, is a substrate of ubiquitin ligase that may promote protein hydrolysis, we classified T13 as being functionally related to senescence. In the *ASK1* mutant of *Arabidopsis thaliana*, vegetative and reproductive

growth, leaf area, cell number, corolla number, and anther number were all markedly decreased. ASK increases plant sensitivity to auxin (Maldonado-Calderon et al., 2012), and it affects the normal separation of alleles in the microsporocyte during the post-meiotic phase, thus causing male sterility in *Arabidopsis* (Yang et al., 1999). Furthermore, ASK plays important roles in corolla and stamen development, and the absence of ASK has adverse effects on embryo formation and postembryonic development (Liu et al., 2004). Based on these characteristics, T13 is likely to be involved in protein hydrolysis, and it is classified as functionally related to senescence. However, the protein hydrolyzed by ASK functions as a gene repressor that promotes growth and development, so T13 may indirectly promote plant growth and development. Relative to the control flowers, T13 expression decreased markedly at both 5 and 48 h after emasculation, indicating that emasculation had a negative effect on flower development, embryo formation, and development during the postembryonic period.

TDFs related to stress responses

T11 and T108 were both homologous to a gene encoding a lipoxygenase inducer. This type of gene plays roles in insect resistance, induction of cell senescence, and responses to mechanical injury to cells (Coffa et al., 2005). T11 was expressed highly in flowers at 5 h after emasculation, and T108 expression increased at 48 h after emasculation. Both T24 and T25 were homologous to a putative disease-resistance protein. The function of the T28 homolog was related to the resistance response of alkaline anaerobic bacteria to oxygen (Wagner et al., 2001). A gene homologous to T66 showed a strong antibacterial effect *in vitro* (Shi et al., 2014). T24, T25, T28, and T66 all exhibited little or no expression at 5 h in the control flowers, but their expression levels were markedly increased in the emasculated flowers.

TDFs related to signal transduction and growth regulation

Genes functionally related to signal transduction and growth regulation accounted for 16.4% of the sequenced TDFs. The expression of many of the TDFs in these categories decreased after emasculation. T39 was expressed highly at 5 and 48 h in the control flowers, but showed very little expression in the emasculated flowers. The T39 homolog, a component of the adaptor protein complex 2 (AP-2), belongs to the cohesion protein complex. AP promotes the formation of vesicles encapsulated by clathrin and participates in the identification of target proteins to be transported (Kelly et al., 2008). AP also promotes the endocytosis of vesicles containing target proteins. Endocytosis of vesicles and the target proteins requires the participation of AP (Owen et al., 2004). The T39 homolog also plays important roles in intracellular transport. The *Arabidopsis* homologs of T63 belong to the BT protein family, which includes auxin-response proteins that play important roles in male and female gametophyte development in *Arabidopsis* (Robert et al., 2009). BT also reduces the inhibition of *Arabidopsis* seed germination by abscisic acid and carbohydrates and increases the plant response to auxin (Mandadi et al., 2009). T63 was highly expressed in control flowers at 5 h, but showed very little expression in the emasculated flowers. T86 and T100 homologs are GTP-binding proteins necessary for the transport of protein and RNA from the nucleus to the cytoplasm. T86 expression decreased at 48 h in the emasculated flowers, and T100 was highly expressed only in control flowers at 48 h. T96 was homologous to the Rab11 gene, which is important in the control of cellular osmotic pressure (Harris et al.,

2001). T96 was expressed highly in control flowers at 5 and 48 h, but expressed at low levels in the emasculated flowers.

The expression of many of the TDFs that were functionally related to signal transduction and growth regulation increased after emasculation. T14 was homologous to a gene encoding a plant microtubule binding protein, which guides the growth direction of cortex microtubules in some organs (Yao et al., 2008). T14 expression increased at 5 h in the emasculated flowers. T22 was homologous to an anticoagulation protein, which promotes the recycling of membrane receptor proteins and plays an important role in the regulation of intercellular signal transduction (Heazlewood et al., 2004). T22 was highly expressed at 5 and 48 h after emasculation, but was expressed at very low levels in the control flowers. The T31 homolog inhibits signal transduction mediated by the epidermal growth factor receptor (Yoo et al., 2004). T31 expression was increased markedly at 5 h in the emasculated flowers relative to the control flowers. T87 was homologous to an auxin-induced protein that may play roles in the connection between the cell wall and the cytoplasm (Neuteboom et al., 1999). T87 was highly expressed only in flowers 5 h after emasculation. T90 was homologous to a calcium binding protein that promotes cell division and trichome formation (Reddy et al., 2004). T90 was highly expressed at 5 h in both the control flowers and emasculated flowers, but exhibited very little expression at 48 h in either.

TDFs related to transcription factors

Three TDFs were homologous to transcription factor genes. T3 was homologous to a transcription factor belonging to the GRAS family, the function of which remains to be determined. T19 was homologous to an NAC transcription factor that promotes cell proliferation, differentiation, and division (Smyczynski et al., 2006). T3 and T19 were both expressed highly at 5 and 48 h after emasculation, but showed little expression in the control flowers. T101 was homologous to a retroelement that might bind nucleic acid and zinc ions. T101 was expressed highly at 5 h after emasculation.

TDFs related to photosynthesis

T53 was homologous to a cytochrome C gene or a gene involved in the transfer of sodium and hydrogen ions. Cytochrome C maintains photosystem stability and promotes the release of oxygen. Sodium and hydrogen ion transfer regulates intracellular pH (Taglicht et al., 1991), but more data are needed to clarify the function of T53. T53 was highly expressed in the control flowers at 5 h, but exhibited little expression in the emasculated flowers.

RT-qPCR analysis

The expression levels of eight TDFs, representing genes that exhibited various expression patterns in response to emasculation damage, were determined by RT-qPCR. The black locust *ACTIN* gene was used as an internal control to which transcript abundance was normalized. The transcript fold-ratios determined by RT-qPCR were similar to those determined by cDNA-AFLP for all eight TDFs, confirming the reliability of the cDNA-AFLP technique (Figure 3).

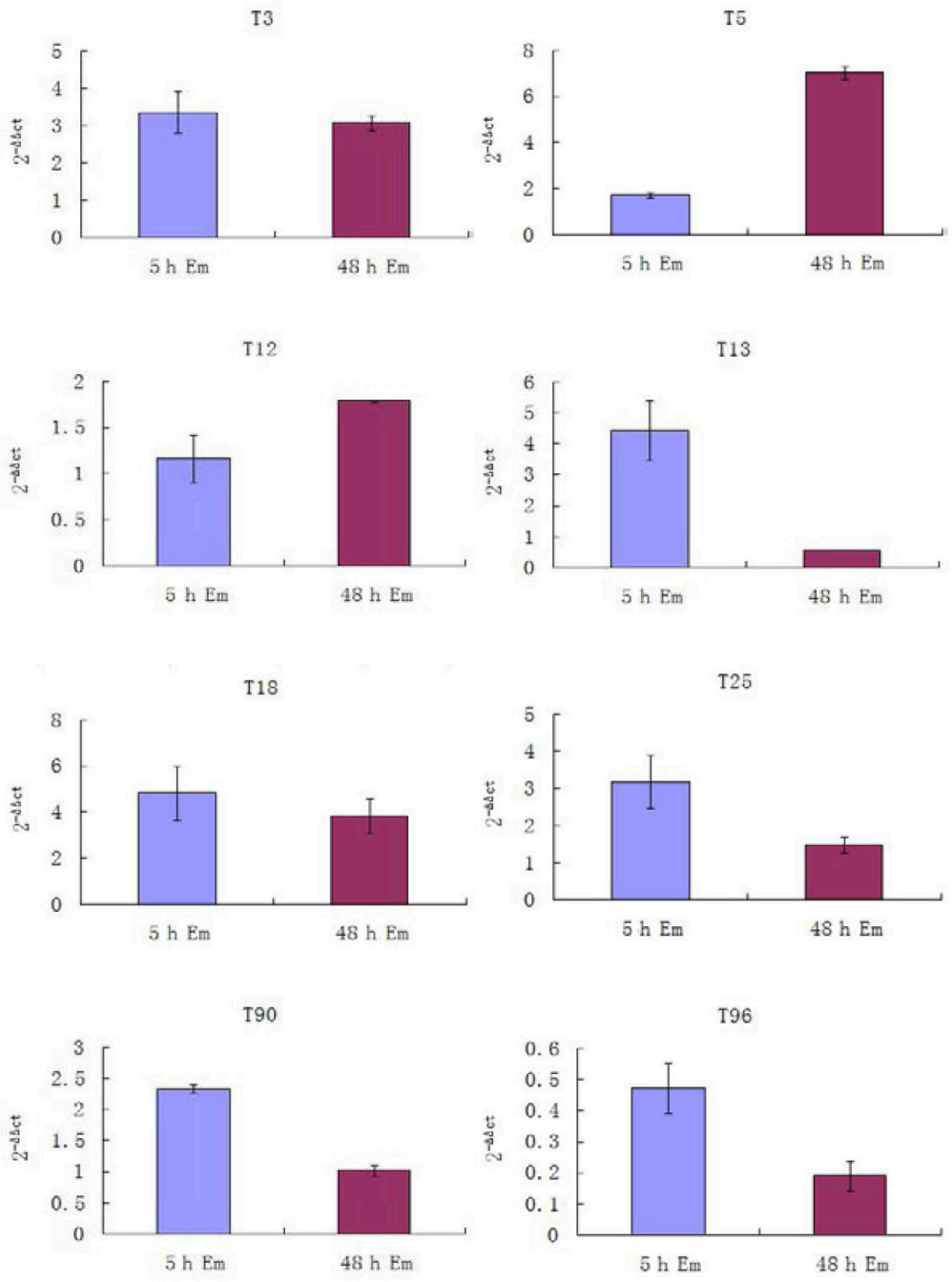

Figure 3. Transcription of eight selected black locust genes at various stages of emasculation (Em) treatment as determined by RT-qPCR.

DISCUSSION

Of the 73 TDFs sequenced, 40 were differentially expressed between emasculated and control flowers at 5 h, and 10 TDFs were differentially expressed at 48 h. Moreover, 21 TDFs were differentially expressed between emasculated and control flowers at both 5 and 48 h. In addition, two genes were expressed at very high levels in both emasculated and control flowers at 5 h, but exhibited no expression at 48 h in either. These results imply that the impact of emasculation on gene expression was greater at 5 h than at 48 h.

Based on our results, the emasculation of black locust flowers has multiple effects on cellular metabolism. Aldolase activity is inhibited, thereby affecting sugar metabolism (Brinkkoetter et al., 2002). The synthesis and metabolism of long-chain fatty acids are affected, which allows flowers to become dry with subsequent negative effects on pollen growth (Trenkamp et al., 2004). The properties of epithelial cells are altered (Pruitt et al., 2000), which hinders the differentiation of pollen and stigma and inhibits epidermal cell growth in ovules (Lolle and Cheung, 1993). Membrane protein metabolism is inhibited, thus affecting the transport of membrane materials. Ribosome function is affected, resulting in the inhibition of protein synthesis. In combination, these effects of flower emasculation adversely affect substance metabolism, energy metabolism, and pollen and ovule development, and they may accelerate the flower ageing process, resulting in a reduced rate of seed set after artificial hybridization.

Not all of the responses of black locust flowers to emasculation were negative. Plants can cope with the stress caused by damage through metabolic changes. Our results showed that the expression of a tubulin-related gene increased in emasculated flowers. The levels of hyaluronic acid increased in emasculated flowers, thereby improving the water retention capacity of the cells and enhancing the maintenance of cell function. Emasculation can promote cell wall synthesis by inducing pectin synthesis, which may play a role in cellular resistance to external stress (Usadel et al., 2004).

Emasculation damage can also promote the synthesis and dephosphorylation of ATP, both of which are important for cellular energy metabolism. Emasculation increased the expression of a 28S large subunit rRNA gene, suggesting that the cells may be able to resist injury-induced stress through enhanced protein synthesis.

The expression of the Xpr1 protein, which is associated with ageing, was upregulated. The Xpr1 protein in combination with retroviruses can induce apoptosis in the human body (Yan et al., 2010), which suggests that emasculation damage may cause susceptibility to external viral infection and accelerate senescence and apoptosis. Emasculation also increased the levels of ubiquitin ligase, which promotes protein degradation.

Emasculation reduced SKP1 gene expression, which could adversely affect flower vegetative and reproductive growth, reduce the responsiveness of flowers to auxin (Lohmann et al., 2010), and impede corolla growth. Decreased SKP1 gene expression would also hinder embryo formation and late embryonic development (Liu et al., 2004). These results suggest that emasculation affects flower development and adversely affects embryo development. These effects may be important contributors to low seed set rates after artificial hybridization.

Lipoxygenase gene expression was induced by emasculation. Lipoxygenase responds to mechanical damage to cells, and it functions in insect resistance and cell ageing (Coffa et al., 2005). Emasculation also upregulates the expression of genes related to antimicrobial, antiviral and antioxidant responses in flower organs. Such changes in gene expression could increase the ability of floral organs to resist damage caused by emasculation, but they may also promote premature ageing.

The expression of proteins involved in growth regulation and signal transduction was affected by emasculation. The expression of a cohesion complex protein was reduced, which could severely suppress cellular protein transport functions (Owen et al., 2004; Kelly et al., 2008). BT protein expression was inhibited, thereby reducing the responsiveness of flower organs to auxin. Based on the functions of TDF homologs we identified from black locust, RNA transport from the nucleus to the cytoplasm, the ability to regulate cellular osmotic pressure, epidermal growth factor receptor signal transduction, and cellular pH regulation would be inhibited by emasculation. These results suggest that the effects of emasculation are detrimental to normal flower development.

Black locust flowers can resist the stress of damage caused by emasculation through reinforcement of a subset of physiological functions. Emasculation promoted the expression of a plant microtubule-binding protein that plays a guiding role in the synthesis of cortical microtubules and the growth of some organs (Yao et al., 2008). The expression of the NAC transcription factor increases in *Arabidopsis* flowers treated with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) or abscisic acid, suggesting that NAC may be associated with flower senescence (De Oliveira et al., 2011). We showed that emasculation increased NAC transcription factor expression in black locust flowers, which indicates that emasculation likely promotes flower senescence.

Our analysis showed that emasculation influences sugar metabolism, protein metabolism, lipid metabolism, energy metabolism, substance transport, resistance signal transduction, osmotic adjustment, pH adjustment, photosynthesis, and other physiological process in flowers. These effects may be important contributors to the premature dropping of emasculated flowers, and they may lead to low seed set rates after artificial hybridization. We also showed that, in addition to negative impacts on flowers, emasculation could also promote some metabolic and signaling processes that enhance the capacity of flowers to resist damage caused by stress.

We sequenced 73 TDFs, including 15 of unknown function. In addition, 17 of the sequenced TDFs had no homologous entries in GenBank that were identifiable using BLASTx and BLASTn searches, indicating that the functions of many genes related to black locust flower emasculation remain unknown. If the functions of these fragments can be clarified in future studies, additional mechanisms of emasculation that affect flower development and seed set rates will be revealed.

Conflicts of interest

The authors declare no conflict of interest.

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