

Article

Effects of Cutting, Pruning, and Grafting on the Expression of Age-Related Genes in *Larix kaempferi*

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Abstract: Grafting, cutting, and pruning are important horticultural techniques widely used in the establishment of clonal forestry. After the application of these techniques, some properties of the plants change, however, the underlying molecular mechanisms are still unclear. In our previous study, 27 age-related transcripts were found to be expressed differentially between the juvenile vegetative (1- and 2-year-old) and adult reproductive (25- and 50-year-old) phases of *Larix kaempferi*. Here, we re-analyzed the 27 age-related transcripts, cloned their full-length cDNA sequences, and measured their responses to grafting, cutting, and pruning. After sequence analysis and cloning, 20 transcription factors were obtained and annotated, most of which were associated with reproductive development, and six (*LaAGL2-1*, *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, *LaAGL11*, and *LaAP2-2*) showed regular expression patterns with *L. kaempferi* aging. Based on the expression patterns of these transcription factors in *L. kaempferi* trees subjected to grafting, cutting, and pruning, we concluded that (1) cutting and pruning rejuvenate the plants and change their expression, and the effects of cutting on gene expression are detectable within 14 years, although the cutting seedlings are still maturing during these years; (2) within three months after grafting, the rootstock is more sensitive to grafting than the scion and readily becomes mature with the effect of the scion, while the scion is not readily rejuvenated by the effect of the rootstock; and (3) *LaAGL2-2* and *LaAGL2-3* are more sensitive to grafting, while *LaAP2-2* is impervious to it. These findings not only provide potential molecular markers to assess the state of plants but also aid in studies of the molecular mechanisms of rejuvenation.

Keywords: larch; vegetative propagation; rejuvenation; gene expression; horticultural technique

1. Introduction

Vegetative propagation plays an important role in establishing clonal forestry, in which grafting and cutting are simple, rapid, and common methods, while pruning is a useful means of promoting the production of elite cutting wood and scions. However, with tree aging and maturing a great decline occurs in the rooting ability of cuttings [1,2]; this is the main limiting factor for the production of cutting seedlings and establishing clonal forestry. Therefore, the reversal of maturity or the maintaining of the juvenile stage are of great practical importance. In addition, grafting, cutting, and pruning are also useful techniques to rejuvenate plants [3,4]. Continuous grafting of mature scions onto juvenile rootstock can be used as a tool to rejuvenate the scions and improve their rooting ability, and this

has been applied in many forest trees [4], such as *Quercus acutissima* [5]. After application of these horticultural techniques, plants change morphologically and physiologically, and show some juvenile characteristics, such as a stronger rooting ability [6,7]; however, the physical and molecular mechanisms underlying rejuvenation are still unclear.

Many genes have been found to function in plant aging, some of which promote maturation, such as *Suppressor of Overexpression of Constans 1 (SOC1)* [8], *APETALA1 (AP1)* [9], *Squamosa-Promoter Binding Protein-Like (SPL)* [10], and *DAL1* (a MADS-box gene) [11], and some delay maturation or maintain the juvenile state, such as microRNA miR156 [12], miR171 [13], *Tempranillo (TEM)* [14], *Terminal Flower 1 (TFL1)* [15], *MdTFL1* and *MdTFL1a* [16], Juvenile-to-Adult transition (*JAT*) [17], *LaAP2L1* (a heterosis-associated AP2/EREBP transcription factor from *Larix*) [18], and *Picea abies APETALA2*-like genes [19]. In our previous study, 27 age-related transcripts were found to be expressed differentially in stems between the juvenile vegetative (1- and 2-year-old) and adult reproductive (25- and 50-year-old) phases of *L. kaempferi*, and some of them are homologous to the genes noted above (Figure 1), indicating that they might have similar functions in aging; meanwhile, they may be useful as molecular markers to determine the state of plants [20]. Together, these findings not only aid studies of the molecular mechanisms of aging and rejuvenation, but also provide candidate genes as molecular markers to assess the state of plants.

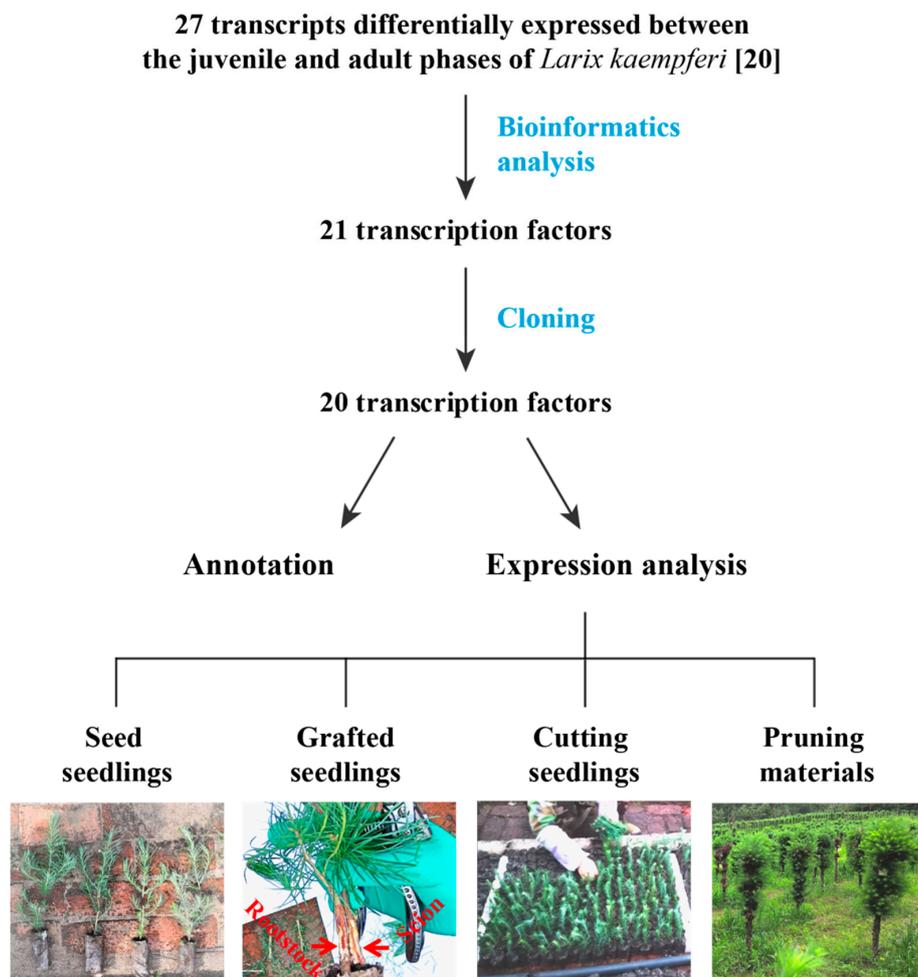


Figure 1. Analytical flowchart. Twenty-seven transcripts differentially expressed between the juvenile vegetative (1- and 2-year-old) and adult reproductive (25- and 50-year-old) phases of *Larix kaempferi*, identified in a previous study [20] were analyzed again and used to identify molecular markers and assess the effects of cutting, grafting, and pruning on gene expression.

In fact, some have already been used as molecular markers. For example, miR156 is a potential marker of juvenility, and *AP1* and *SOC1* may be markers of the reproductive phase transition in avocado, mango, and macadamia plants [21]. However, the markers previously reported have generally been used to identify the state of naturally growing plants, and there has been limited use for identifying the state of plants treated with various horticultural techniques. Recently, grafting effects on gene expression at the transcriptome level have been reported in walnut [22] and tea [23] plants, providing more candidate molecular marker genes.

Here, we analyzed 27 age-related transcription factors again, cloned their full-length cDNA sequences, and measured their responses to grafting, cutting, and pruning to (1) determine if they can be used as molecular markers to determine the state of *L. kaempferi* trees treated with these horticultural techniques, and (2) study the molecular mechanisms of rejuvenation (Figure 1).

2. Materials and Methods

2.1. Plant Materials

The lateral branches produced in the current year from the tops of *L. kaempferi* trees were collected in early July of 2018 and 2019. The trees were located in Dagujia seed orchard (42°22' N, 124°51' E), Liaoning Province in Northeast China, and were produced from seeds, grafted seedlings, and cutting seedlings (Table 1). Rootstocks and scions from the grafted seedlings were sampled separately (Table 1). Pruning materials were sampled from four special cutting seedlings, which were pruned (hedged or sheared) and set at 1.5 m high (Table 1). After the removal of branches and needles, the stems from at least three of each kind of tree or each age category were pooled, frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

Table 1. Information on samples.

Samples	Age	Other Information
Seed Seedlings	1 year	These seedlings were grown from seeds
	3 years	
	5 years	
	7 years	
	9 years	
	11 years	
	13 years	
	14 years	
Grafted Seedlings	3 months	Rootstocks were from 1-year-old and scions from 12-year-old seed seedlings
Cutting Seedlings	1 year	Cuttings were sampled from 12-year-old seed seedlings
	2 years	
	3 years	-
	5 years	-
	7 years	-
	9 years	-
	11 years	-
	13 years	-
Pruning Materials	14 years	Cuttings were sampled from 8-year-old seed seedlings
		Cutting seedlings were pruned; they were propagated from cuttings from 8-year-old seed seedlings

- indicates no age information.

2.2. RNA Extraction and cDNA Synthesis

Total RNA was extracted with the EasyPure RNA Kit (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. A 2.5 µg aliquot of total RNA was reverse-transcribed into cDNA with

the TransScript II One-step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China), and subsequently diluted for gene isolation and expression analysis.

2.3. Sequence Analysis, Full-Length cDNA Cloning, and Annotation

The open reading frame (ORF) finder (<https://www.ncbi.nlm.nih.gov/orffinder/>) was used to identify the ORFs of the 27 transcription factors reported in our previous work [20] (Table 2). Based on the results, primers (Table S1) were designed to clone their full-length cDNA sequences (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) with Platinum[®] Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The PCR products were purified with a gel extraction kit (Tiangen, Beijing, China), ligated into the pEASY[®]-T1 simple cloning vector (TransGen Biotech, Beijing, China), and sequenced. The full-length cDNA sequences were submitted to GenBank (Table 2). Based on the results of blast analysis with *Arabidopsis thaliana* homologs, we assigned the Gene Ontology (GO) terms associated with the *A. thaliana* homologs to larch transcription factors.

Table 2. Analysis of 27 transcripts expressed differentially between juvenile vegetative (1- and 2-year-old) and adult reproductive (25- and 50-year-old) phases of *Larix kaempferi* [20].

Family	Name	Transcript ID	Accession Number
MADS-box	<i>LaCAL</i>	comp81209_c0_seq1	MN790743
	<i>LaAGL2-1</i>	comp125095_c0_seq5	MN790744
	<i>LaAGL2-2</i>	comp126977_c0_seq13	MN790745
		comp125095_c0_seq9	
		comp126977_c0_seq2	
	<i>LaAGL2-3</i>	comp126977_c0_seq16	MN790746
		<i>LaSOC1-1</i>	comp128412_c0_seq11
	<i>LaSOC1-2</i>	comp128412_c0_seq23	MN790748
		<i>LaSOC1-3</i>	comp128412_c0_seq8
	comp128471_c0_seq5		
	comp128471_c0_seq24		
	<i>LaSOC1-4</i>	comp129709_c0_seq16	MN790750
		NC	
	<i>LaAGL1</i>	comp129017_c0_seq15	MN790751
	<i>LaAGL11</i>	comp128471_c0_seq14	MN790752
<i>LaAGL42</i>	comp128471_c0_seq18	MN790753	
ERF	<i>LaERF017</i>	comp124322_c0_seq3	MN790754
	<i>LaERF3</i>	comp129386_c0_seq9	MN790755
GRAS	<i>LaSCL29</i>	comp114072_c0_seq3	MN790756
AP2	<i>LaAP2-1</i>	comp128327_c0_seq16	MN790757
	<i>LaAP2-2</i>	comp122930_c0_seq2	MN790758
DOF	<i>LaHCA2</i>	comp120092_c0_seq3	MN790759
C3H	<i>LaOZF2</i>	comp111742_c0_seq2	MN790760
MYB_Related	<i>LaTRFL6</i>	comp128112_c0_seq2	MN790761
G2-Like	<i>LaPHL1</i>	comp130729_c0_seq1	MN790762

NC indicates that the full-length cDNA for this transcript was not cloned successfully. *LaCAL*, *Larix kaempferi* CAULIFLOWER; *LaAGL2-1*, *L. kaempferi* AGAMOUS-Like 2-1; *LaAGL2-2*, *L. kaempferi* AGAMOUS-Like 2-2; *LaAGL2-3*, *L. kaempferi* AGAMOUS-Like 2-3; *LaSOC1-1*, *L. kaempferi* SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1-1; *LaSOC1-2*, *L. kaempferi* SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1-2; *LaSOC1-3*, *L. kaempferi* SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1-3; *LaSOC1-4*, *L. kaempferi* SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1-4; *LaAGL1*, *L. kaempferi* AGAMOUS-Like 1; *LaAGL11*, *L. kaempferi* AGAMOUS-Like 11; *LaAGL42*, *L. kaempferi* AGAMOUS-Like 42; *LaERF017*, *L. kaempferi* Ethylene-Responsive Transcription Factor ERF017; *LaERF3*, *L. kaempferi* Ethylene-Responsive Transcription Factor 3; *LaSCL29*, *L. kaempferi* Scarecrow-Like 29; *LaAP2-1*, *L. kaempferi* APETALA 2-1; *LaAP2-2*, *L. kaempferi* APETALA 2-2; *LaHCA2*, *L. kaempferi* High Cambial Activity 2; *LaOZF2*, *L. kaempferi* Oxidation-Related Zinc Finger 2; *LaTRFL6*, *L. kaempferi* TRF-Like 6; *LaPHL1*, *L. kaempferi* PHR1-Like 1.

2.4. Quantitative Reverse Transcription Polymerase Chain Reaction

The quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to assay the expression patterns of the identified transcription factors in different samples, with *Larix kaempferi* fructose-1,6-bisphosphatase 1 (*LaFBP1*, GenBank accession no. MN790763) (Table S2), which is expressed stably during tree aging [24], as the internal control. The qRT-PCR analysis was performed on a Bio-Rad CFX96 PCR system, using a TB Green® Premix Ex Taq™ (Tli RNase H Plus) (Takara, Shiga, Japan). Each reaction was carried out on 2 µL of diluted cDNA sample, in a total reaction system of 25 µL. The reaction procedure was set up according to the manufacturer's protocol: 95 °C for 30 s, then 40 cycles at 95 °C for 5 s, and at 60 °C for 30 s, followed by a melting step from 65 to 95 °C. The relative expression ratio was expressed using the $2^{-\Delta\Delta C_t}$ method. The expression level of each gene was standardized to the constitutive expression level of *LaFBP1*. The ratio between the expression levels of each age-related gene and *LaFBP1* for each sample was calculated using the relative quantitative analysis method. The sample with the minimum expression level was used as a calibrator and was set to a value of 1. The qRT-PCR was performed with three technical replicates, and the data are shown as the mean \pm SD.

3. Results and Discussion

3.1. Cloning and Annotation of 20 Transcription Factors

After analyzing the sequences of 27 transcription factors, we found that some had the same ORF (Table 2); in total, 21 non-redundant sequences were obtained, while 20 were cloned successfully and used for further study (Table 2). Blast analysis of the 20 cloned sequences was performed again. The results showed that the 20 transcription factors were from eight families and had 14 homologs in *A. thaliana* (Table 2). Among the eight families, the MADS-box family had the most members (Table 2).

Based on the names and GO annotation of their homologs in *A. thaliana*, they were designated and annotated (Table 2). In total, 36 GO terms in the biological process category were obtained, among which 18 were associated with processes of plant reproductive development and their hormonal and environmental control (Figure 2), such as “flower development”, “meristem maintenance”, “response to gibberellin”, and “vernalization response”, and therefore provided more information about the roles of these 20 transcription factors in the sexual reproductive development of *L. kaempferi*. However, further experiments were required to assess whether they showed regular expression patterns with aging and if they could be used as molecular markers to assess the state of plants.

3.2. Expression Patterns of 20 Transcription Factors during Larch Tree Aging

The qRT-PCR assays were used to reveal the expression patterns of the 20 transcription factors in 1-, 3-, 5-, 7-, 9-, 11-, and 13-year-old trees. We found that the expression level of *LaAP2-2* decreased with age (Figure 3o), while that of *LaAP2-1* decreased, and that of *LaOZF2* increased from 1 to 5 years (Figure 3n,q) and the expression levels of *LaAGL2-1*, *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, *LaSOC1-3*, *LaAGL11*, and *LaAGL42* increased from 1 to 7 years, while almost no changes were found in the expression levels of *LaAGL2-1*, *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, *LaAGL11*, and *LaAGL42* from 7 to 13 years and a decrease occurred in the expression level of *LaSOC1-3* (Figure 3).

The maturity status of a plant is dynamic and relative and can be reflected by many aspects of tree growth and development, including the changes in gene expression, morphology, and physiology. For example, in larches the capacity to produce seeds occurs at about 10-years-old, the effect of donor tree age on rooting ability is obvious, and at about 12-years-old rooting ability decreased greatly [25,26]. Based on the regular expression patterns of *LaAGL2-1*, *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, *LaAGL11*, and *LaAP2-2* over the course of aging up to 13-years-old, we concluded that their expression is age-dependent. Given that the maturity status of a plant changes after the application of horticulture techniques, we speculated that the expression of these six age-related genes might reflect this change.

Next, we measured the expression patterns in trees subjected to grafting, cutting, and pruning to determine the effects of these techniques on the expression of these genes.

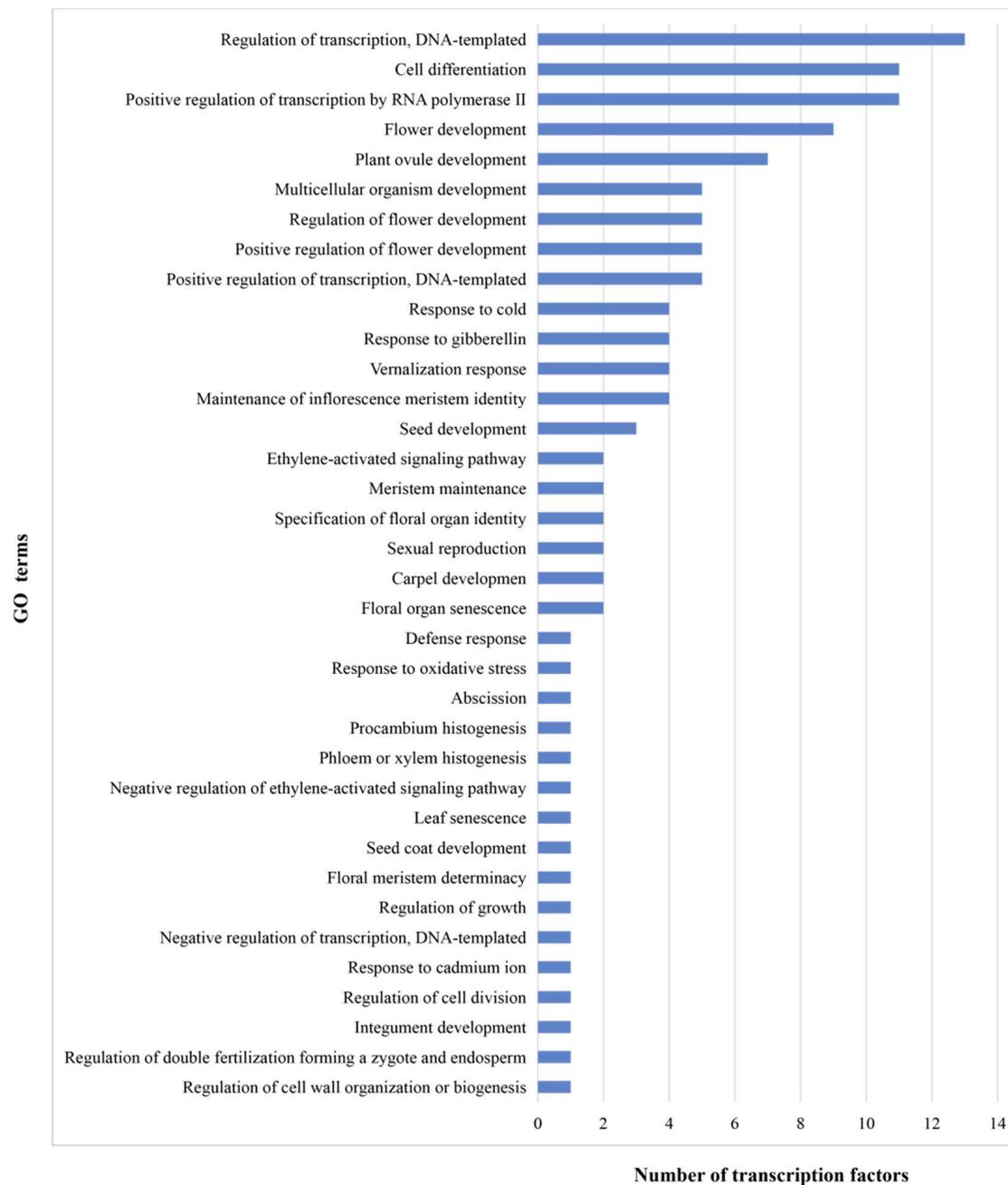


Figure 2. Biological processes in Gene Ontology (GO) terms. Based on the results of blast analysis, the GO terms associated with the *Arabidopsis thaliana* homologs were assigned to the *Larix kaempferi* transcription factors.

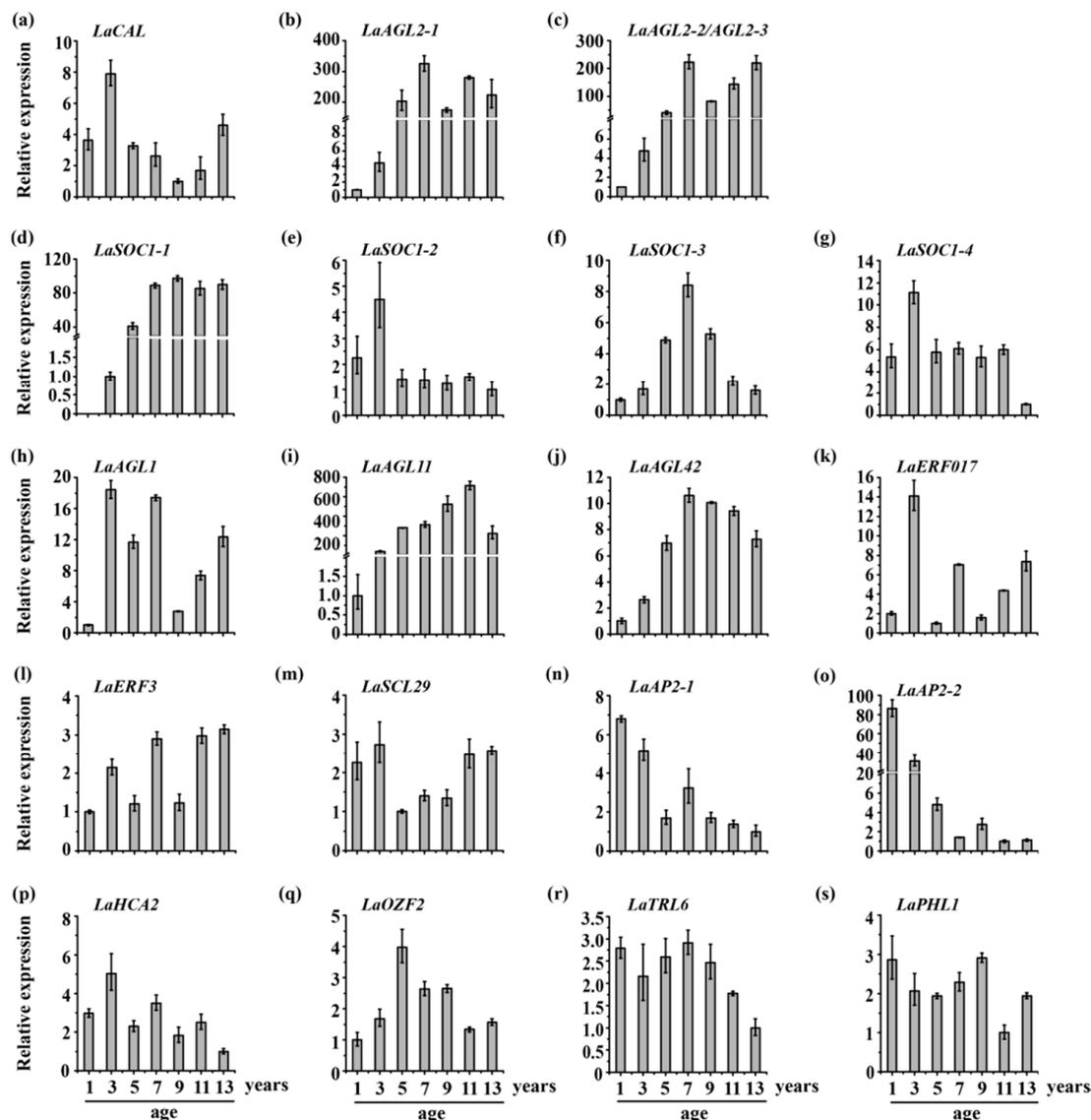


Figure 3. Expression patterns of 20 transcription factors [*LaCAL* (a), *LaAGL2-1* (b), *LaAGL2-2* (c), *LaAGL2-3* (c), *LaSOC1-1* (d), *LaSOC1-2* (e), *LaSOC1-3* (f), *LaSOC1-4* (g), *LaAGL1* (h), *LaAGL11* (i), *LaAGL42* (j), *LaERF017* (k), *LaERF3* (l), *LaSCL29* (m), *LaAP2-1* (n), *LaAP2-2* (o), *LaHCA2* (p), *LaOZF2* (q), *LaTRL6* (r), *LaPHL1* (s)] in 1-, 3-, 5-, 7-, 9-, 11-, and 13-year-old *Larix kaempferi* seed seedlings ($n \geq 6$, sampled in 2019) assayed by qRT-PCR with *LaFBP1* as the internal control.

Here, the changes in gene expression were emphasized and we tried to correlate the changes in gene expression with those in physiological status, especially flowering and rooting ability. *DAL1*, a MADS-box transcription factor from *P. abies*, is expressed increasingly from the age of 5-years-old, and its over-expression in *Arabidopsis* results in early flowering [11]; here the putative homolog of *DAL1*, *LaAGL2*, was identified and showed almost the same expression pattern as *DAL1* (Figure 3), indicating its involvement in the transition from the juvenile vegetative to the adult reproductive phase. While the capacity to produce seeds occurs at about 10-years-old in *Larix*, the molecular regulatory mechanism for the vegetative to reproductive development in trees mediated by *LaAGL2* is still unclear. Regarding rooting ability, it has been detected that the rooting rate of larch cutting decreased sharply at about 12-year-old [25]; further work is needed to understand how the age-related genes function in this regenerative process.

3.3. Effects of Pruning and Cutting on the Expression of Six Age-Related Genes

In 14-year-old seed seedlings, we found higher expression levels of *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, and *LaAGL11* and lower levels of *LaAP2-2* (Figure 4). Compared with 14-year-old seed seedlings, cutting seedlings showed a decrease in the expression levels of *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, and *LaAGL11* and an increase in the level of *LaAP2-2*, while almost no change was found in the level of *LaAGL2-1* (Figure 4). After pruning the cutting seedlings, clear changes were found in the expression levels where *LaAGL2-1*, *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, and *LaAGL11* transcript levels decreased and *LaAP2-2* transcript levels increased (Figure 4). These results suggested that the expression of these six age-related genes is affected by cutting or pruning; based on their expression patterns, we concluded that cutting and pruning rejuvenates plants, and this is indicated by the increased rooting ability of cuttings [27–31].

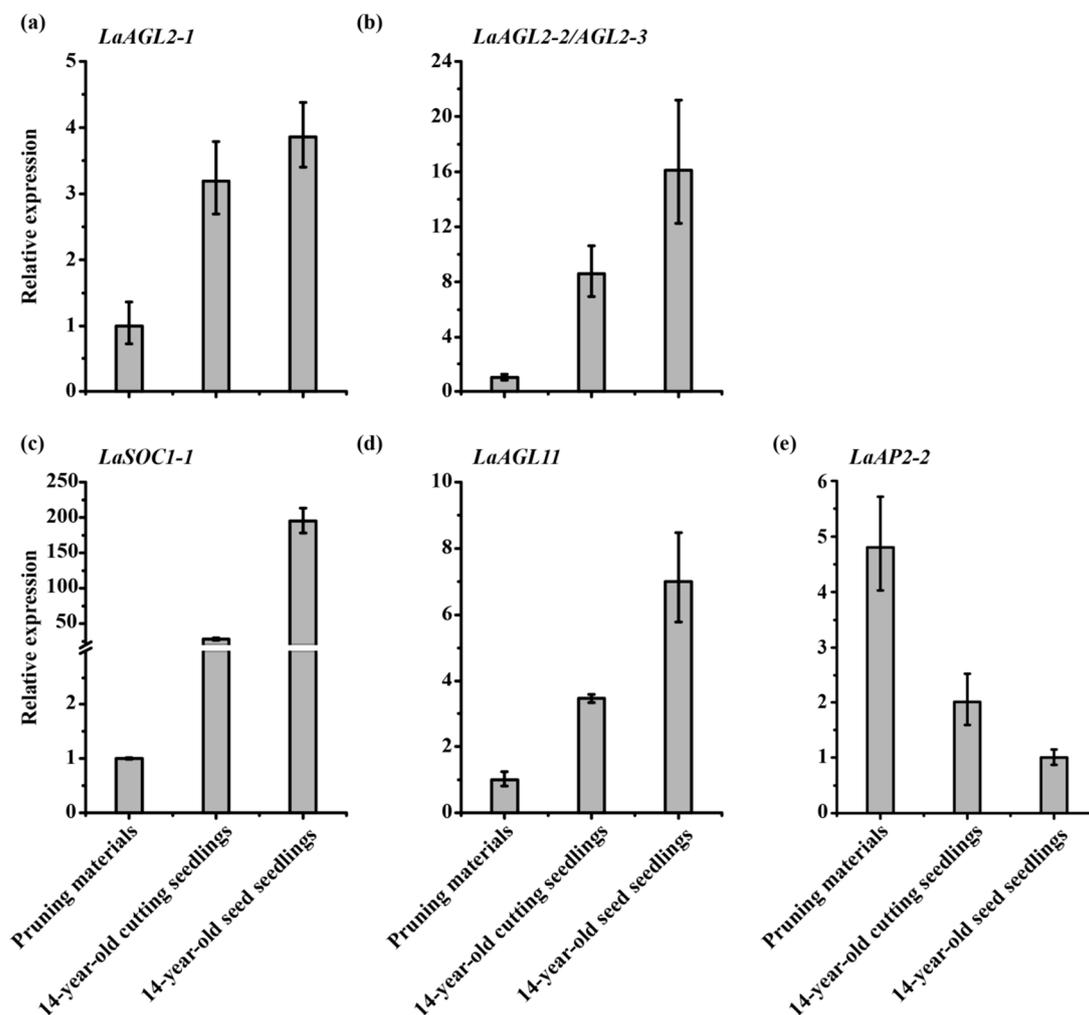


Figure 4. Expression patterns of *LaAGL2-1* (a), *LaAGL2-2* (b), *LaAGL2-3* (b), *LaSOC1-1* (c), *LaAGL11* (d), and *LaAP2-2* (e) in 14-year-old *Larix kaempferi* pruning materials ($n = 3$, sampled in 2018), cutting seedlings ($n = 3$, sampled in 2018), and seed seedlings ($n = 3$, sampled in 2018) assayed by qRT-PCR with *LaFBP1* as the internal control.

Notably, the expression levels of *LaAGL2-1*, *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, and *LaAGL11* in the pruning materials were lower than those in cutting seedlings, and the expression levels of *LaAP2-2* were higher than those in cutting seedlings (Figure 4). These results suggested that pruning further affects the expression of these six transcription factors in plants originating from cuttings. Together, we

concluded that cutting rejuvenates plants, pruning maintains their juvenility [32], and the effect of cutting on gene expression can be detected 14 years later.

To further study the effect of cutting on the expression of the six age-related genes in the subsequent early years after cutting, we assessed their expression patterns in 1- and 2-year-old cutting seedlings. Compared with those in 13- and 14-year-old seed seedlings, from which the cuttings were produced in 2018 and 2017, respectively, the expression levels of *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, and *LaAGL11* decreased in the 1- and 2-year-old cutting seedlings, while the expression level of *LaAP2-2* increased. Almost no change in the expression level of *LaAGL2-1* was found in 1-year-old cutting seedlings and 13-year-old seed seedlings, while it was lower in the 2-year-old cutting seedlings than that in 14-year-old seed seedlings (Figure 5). These results showed that cutting changes the expression of these genes immediately after the treatment and rejuvenates the plant within two years after cutting.

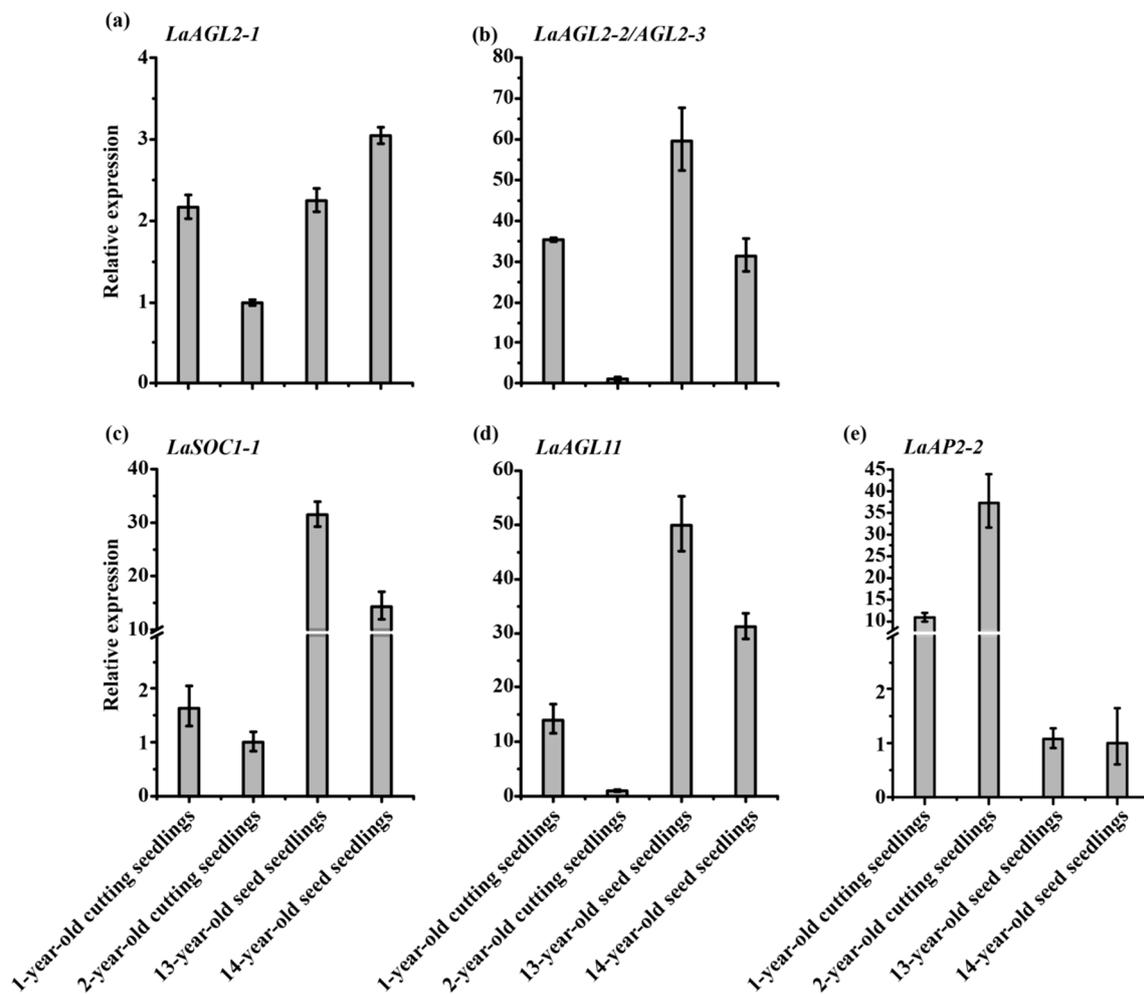


Figure 5. Expression patterns of *LaAGL2-1* (a), *LaAGL2-2* (b), *LaAGL2-3* (b), *LaSOC1-1* (c), *LaAGL11* (d), and *LaAP2-2* (e) in 1- and 2-year-old *Larix kaempferi* cutting seedlings ($n \geq 12$, sampled in 2019) and 13- and 14-year-old seed seedlings ($n \geq 6$, sampled in 2019) assayed by qRT-PCR with *LaFBP1* as the internal control. In 2018 and 2017 1- and 2-year-old cutting seedlings were propagated from 12-year-old seed seedlings that were sampled in 2019 when they were 13- and 14- years old, respectively.

Moreover, we found that the expression levels of *LaAGL2-1*, *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, and *LaAGL11* in 2-year-old cutting seedlings were lower than those in 1-year-old cutting seedlings, and the opposite pattern occurred in *LaAP2-2* expression (Figure 5 and Figure S1). These results indicated that cutting affects the expression of the six age-related genes and these effects are enhanced as the cutting seedling ages.

It is known that as cutting seedlings age, the phenotypes of maturation also appear, so the question arose as to when maturation begins and when the enhancement of gene expression by the cutting effect ends. We set out to answer these questions by assessing the expression patterns of the six age-related genes in 1-, 3-, 5-, 7-, 9-, 11- and 13-year-old cutting seedlings. The lowest levels of *LaAGL2-1*, *LaAGL2-2*, and *LaAGL2-3* transcripts were found in 3-year-old cutting seedlings after which the levels of these transcripts increased; the expression levels of *LaAGL2-1* at 11-years-old, *LaAGL2-2* and *LaAGL2-3* at 9-years-old were almost the same as those at 1-year-old (Figure 6). These data indicated that, in response to cutting, the transcription of *LaAGL2-1*, *LaAGL2-2*, and *LaAGL2-3* in cutting seedlings is suppressed at first, and with aging it is induced again (Figure 6). Based on the expression patterns of these transcripts, we concluded that the enhancement of gene expression by the cutting effect persists for only 2–3 years and maturation begins early. The irregular expression patterns of *LaSOC1-1*, *LaAGL11*, and *LaAP2-2* during the aging of cutting seedlings cannot yet be explained (Figure 6), but these data suggested that the effects of cutting on the expression of age-related genes are complex and regulated by currently unknown mechanisms.

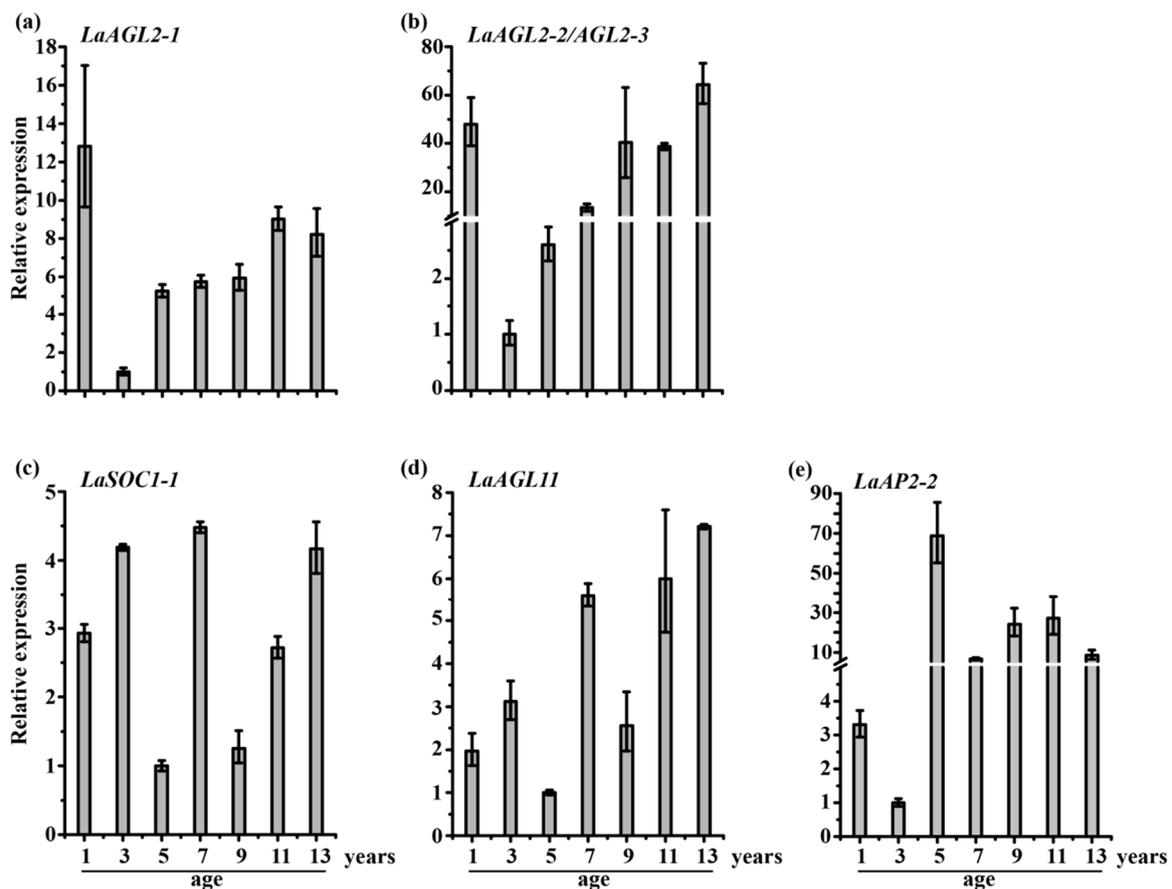


Figure 6. Expression patterns of *LaAGL2-1* (a), *LaAGL2-2* (b), *LaAGL2-3* (b), *LaSOC1-1* (c), *LaAGL11* (d), and *LaAP2-2* (e) in 1-, 3-, 5-, 7-, 9-, 11-, and 13-year-old *Larix kaempferi* cutting seedlings ($n \geq 5$, sampled in 2019) assayed by qRT-PCR with *LaFBP1* as the internal control.

Based on the expression patterns of these age-related genes, we concluded that cutting rejuvenates the plant and affects gene expression; this effect is detected within 14 years, although the cutting seedlings are still maturing during these years.

3.4. Expression Patterns of the Six Age-Related Genes after Grafting

Grafting is also a useful technique that can promote the rejuvenation of many plant species, such as *Sequoia sempervirens* and the European larch [33–35]. Here, we also assessed the effect of grafting on the expression of the six age-related genes in scions and rootstocks. Compared with those in rootstocks, higher expression levels of *LaAGL2-1*, *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, and *LaAGL11*, and a lower level of *LaAP2-2* were found in scions three months after grafting (Figure 7). Compared with those in 1-year-old seed seedlings, higher levels of *LaAGL2-1*, *LaAGL2-2*, *LaAGL2-3*, and *LaSOC1-1* transcripts were found in the rootstocks, while almost no change was found in the levels of *LaAGL11* and *LaAP2-2* (Figure 7), showing that in rootstock, grafting increases the expression of *LaAGL2-1*, *LaAGL2-2*, *LaAGL2-3*, and *LaSOC1-1*, but has no effect on the expression of *LaAGL11* and *LaAP2-2*. Compared with those in 13-year-old seed seedlings, higher expression levels of *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, and *LaAGL11* were found in the scions, while almost no change was found in the levels of *LaAGL2-1* and *LaAP2-2* (Figure 7), showing that in scions, grafting increases the expression of *LaAGL2-2*, *LaAGL2-3*, *LaSOC1-1*, and *LaAGL-11*, but does not affect the expression of *LaAGL2-1* and *LaAP2-2*. Together, these results showed that grafting affects the expression of age-related genes in the rootstock and scion and each gene has a different response to grafting.

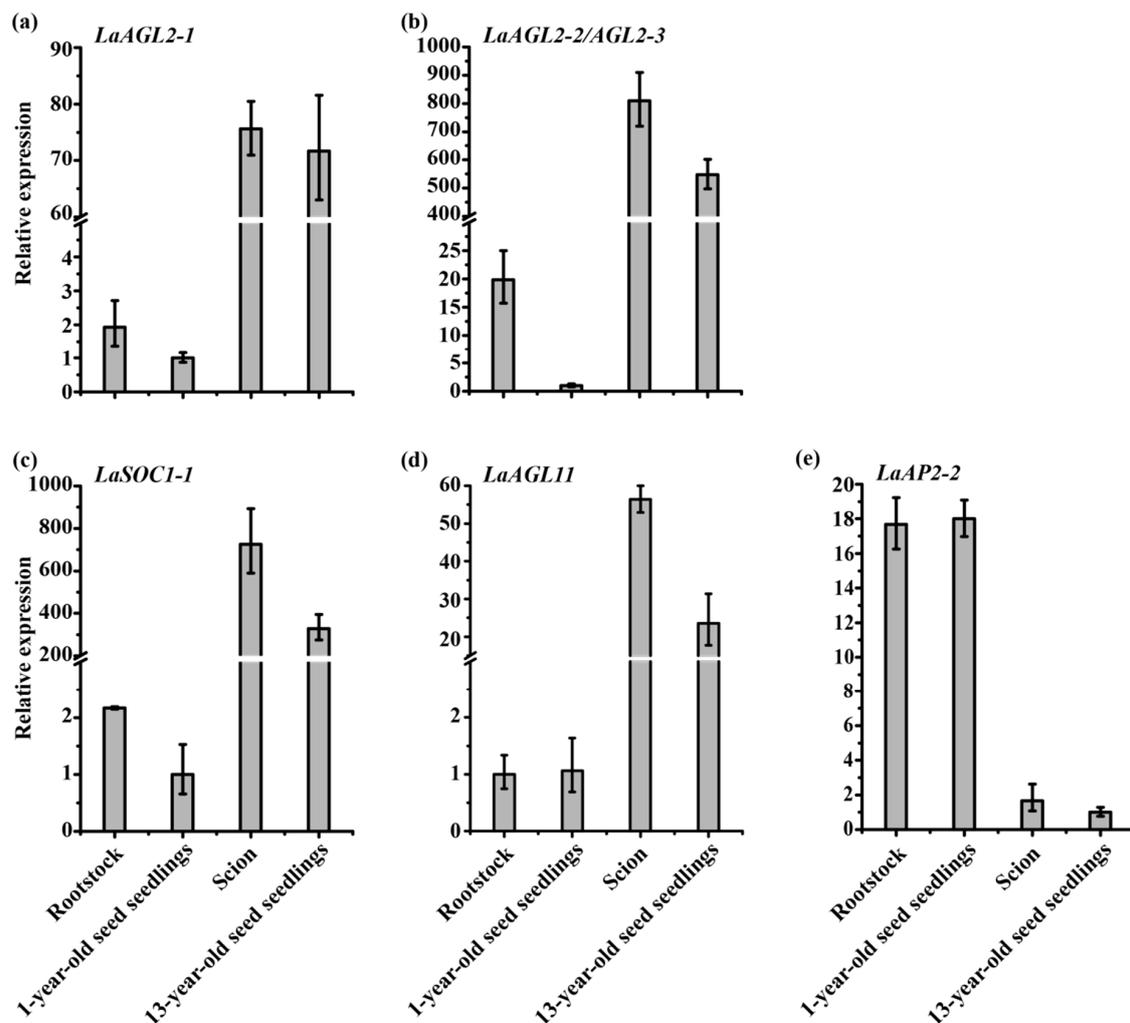


Figure 7. Expression patterns of *LaAGL2-1* (a), *LaAGL2-2* (b), *LaAGL2-3* (b), *LaSOC1-1* (c), *LaAGL11* (d), and *LaAP2-2* (e) in 3-month-old grafted seedlings ($n = 6$, sampled in 2019) and 1- and 13-year-old seed seedlings ($n = 11$, sampled in 2019) assayed by qRT-PCR with *LaFBP1* as the internal control.

Based on the expression patterns of these age-related genes, we concluded that (1) within three months after grafting, the rootstock is more sensitive to grafting than the scion—the rootstock readily matures with the effect of the scion, while the scion is not readily rejuvenated by the effect of the rootstock, because the expression of *LaAGL2-1*, *LaAGL2-2*, *LaAGL2-3*, and *LaSOC1-1* increased in the rootstock and did not decrease in the scion; (2) *LaAGL2-2* and *LaAGL2-3* are more sensitive to grafting than the other four genes because greater changes in their expression occurred after grafting; and (3) *LaAP2-2* is impervious to grafting, because almost no changes in its expression were detected in rootstock and scion.

4. Conclusions

Taken together, we conclude that grafting, cutting, and pruning change the expression of age-related genes and rejuvenate plants through currently unknown mechanisms. The identification of age-related genes helps to reveal the mechanisms of rejuvenation and the understanding of other physiological changes that occur with age, which promotes the establishment of clonal forestry.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/11/2/218/s1>, Figure S1: Expression patterns of *LaAGL2-1* (a), *LaAGL2-2* (b), *LaAGL2-3* (b), *LaSOC1-1* (c), *LaAGL11* (d), and *LaAP2-2* (e) in 1- and 2-year-old *Larix kaempferi* cutting seedlings ($n = 3$, sampled in 2018) assayed by qRT-PCR with *LaFBP1* as the internal control. In 2017 and 2016 1- and 2-year-old cutting seedlings were propagated from 21- and 20-year-old seed seedlings that were sampled in 2018 when they were 22 years old, respectively. The qRT-PCR was performed with three technical replicates, and the data are shown as the mean \pm SD. Table S1: Primers used for cloning 20 transcription factors, Table S2: Primers used for qRT-PCR.

Author Contributions: Y.Z. carried out the study, analyzed the data, and wrote the manuscript. Q.-L.Z. helped to analyze the data and revised the manuscript. W.-F.L. conceived and designed the study, analyzed the data and revised the manuscript. L.-W.Q. and S.-Y.H. provided suggestions on the experimental design and analyses. All authors have read and approved the manuscript.

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