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Full Length Article

Time Series Analysis of Differential Expression Transcript in Four Developmental Phases of Germinating Tobacco Seed

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Abstract

Imbibition, embryonic peripheral tissue rupture and radicle emergence are generally considered as key development stages of germinating tobacco seed. However, the similarities and differences of metabolism among them are yet to be explored. Present study investigated the transcriptomes distinctions at various developmental stages in tobacco seed. The results showed that 2730 and 2708 transcripts were significantly up-regulated during imbibition and radicle emergence respectively, which were significantly higher than that of 1969 during endosperm rupture. Thirteen patterns of short time-series expression profiles (STEM) were found to be significantly clustered, including the patterns of three stages continuously up-regulated during imbibition, endosperm rupture and radicle emergence, any two or only one of up-regulated, but not patterns of continuously or any two stages of down-regulated, even one stage of down-regulated pattern clustered was just focused on the endosperm rupture. Of them, we focused on the continuously up-regulated pattern in this study and noticed 221 transcripts enriched in this pattern, which are participated in ABA signal pathway, cell wall hydrolysis, and light signal transduction metabolism. In addition, the methionine metabolism was stimulated after imbibition, cell division, growth and differentiation were significantly increased prior to endosperm rupture, and photosynthesis rise significantly in radicle emerged seeds. This study preliminarily revealed the metabolic differences among four developmental stages during seed germination in tobacco. © 2020 Friends Science Publishers

Key words: Tobacco seed; Germination; Seed development; Transcriptome; Short time-series expression miner

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Introduction

Seed germination is a complex biological process, and it begins with water absorption and ends at radicle emergence (Bewley, 1997). In tobacco, seed germination can be divided into four development stages as seed imbibition, testa rupture, endosperm rupture and radicle emergence in accordance with the chronological order (Manz *et al.*, 2005). It is evident that the testa rupture and endosperm rupture are respective events, and both of them are the limiting factors for germination (Leubner-Metzger, 2003). Class I β-1,3-glucanase (βGlu I) promotes the endosperm loose and radicle protrusion, enzyme activity of which accumulates just prior to endosperm rupture, but after testa rupture (Leubner-Metzger *et al.*, 1995).

Water uptake in seed germination of tobacco can be divided into three stages, rapid physical imbibition of the first stage, smooth absorption of the second stage, and third active stage begins after endosperm rupture (Manz *et al.*, 2005). Tobacco seeds are oilseeds, and oil mobilization of the seeds occurred before testa rupture and whereafter that not further mobilization (Manz *et al.*, 2005). Gibberellin acid, the key plant hormone that promotes germination and its function probably occurred after the endosperm rupture and before radicle emergence (Gallardo *et al.*, 2002; Ogawa *et al.*, 2003). Hereinabove, seed development during germination in tobacco had been extensively studied. But little is known about the regulatory networks difference in the germinating seeds at different developmental stages.

High-throughput sequencing technology seems to be an effective method to study molecular mechanism of seed germination. In fact, transcriptome (Nakabayashi *et al.*, 2005; Carrera *et al.*, 2008), proteome (Gallardo *et al.*, 2001; Fercha *et al.*, 2013), metabolome (Fait *et al.*, 2006), and multi-omics methods (Kubala *et al.*, 2015) have been exploited to study the molecular network of seed germination. The crops refer to rice (Yang *et al.*, 2007), maize (Fu *et al.*, 2011), wheat (Yu *et al.*, 2014), barley (Potokina *et al.*, 2002), and so on. The organs studied ranged from embryo (Yu *et al.*, 2016), endosperm (Yu *et al.*, 2016) and hypocotyl (Jimenez-Lopez *et al.*, 2011), and so on. Recently, time series analysis of differential expression

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genes has been exploited to study the seed development during germination at the omics level (Yu *et al.,* 2014; Kawakatsu *et al.,* 2017); however, differences in the physiological development stages of germinating seed are rarely considered.

Tobacco is chosen for this study because it is an established model system for germination of endospermic seed, the developmental stage of which can be obviously separated during germination. In this study, dry seeds, imbibed seeds, endosperm rupturing seeds and radicle emerging seeds were comparatively studied for their differences in transcriptome. On this basis, time series analysis of differential expression genes of seeds at four developmental stages was carried out, to analyze the dynamics of geminating seed at transcriptome level. In addition, specific signal pathways in whole and each developmental stage were also explored.

Materials and Methods

Seed Germination, βGlu I Activity Determination and Transcriptome Sample Preparation

The fresh seeds of tobacco were stored till after-ripening and then been used for germination test. Three replicates of 100 seeds in each were cultivated in petri dishes under a 12 h light/dark alternant cycle at 26°C. The germinated seeds were recorded every 12 h, and 0, 36, 72, 108, 144 hs germinating seeds were sampled to quantify the βGlu I activity (Li *et al.,* 2016). According to kinetic curves both of radicle emergence and βGlu enzyme activity, the time points of endosperm rupture and radicle emergence were determined separately after 72nd and 96th h of imbibition. The imbibed, endosperm rupturing and radicle emerging seeds were collected respectively, and together with the dry seeds were prepared for transcriptome sequencing.

RNA Sequencing and Time Series Clustering Analysis of the Sequencing Data

Total RNA of four samples were extracted and mRNAs were enriched from 5.0 ug total RNA. Enriched mRNA were fragmented and purified. cDNAs of which were synthesized, purified, and performed as templates to generate sequencing libraries. The libraries were sequenced using the Illumina HiSeq 2000 platform, and each sample yielded 10 Gb data. The raw reads were cleaned by removing adapter sequences and low-quality bases at 3′ end. The clean reads were then mapped to the genome of tobacco by TopHat (Sierro *et al.,* 2014).

The process of time series clustering analysis (TSCA) was as follows: Firstly, the FPKM method was used to homogenize the data from the four developmental stages. Secondly, the transcripts selected for TSCA need to satisfy two conditions (1. The average FPKM of the expression gene in the four developmental stages was greater than 1.0. 2. There was at least one group that DEseq analysis has significant differences). Finally, the Short time-series Expression Miner (STEM) program was used to cluster analysis of the data from the four development stages, and the significance level of clustering was set to 0.05 and the minimum correlation was set to 0.70. The GOATOOLS program was exploited to pathway enrichment analysis, the procedure using hypergeometric inspection enrichment analysis, and the significance level is set to Bonferroni correction $P \leq 0.05$.

Real-time qPCR

Ten differentially expressed transcripts were selected randomly to verify the result of RNA sequencing. *L25* was selected as the internal reference, and the reaction system were performed according to our published method (Li *et al.,* 2016). The relative expression of these genes were calculated by e $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Correlation analysis was performed on relative expression level of qRT-PCR and FPKM values of RNA-seq, and the accuracy of RNA-seq sequencing results was analyzed based on correlation coefficients.

Results

Time Point of Endosperm Rupture and Radicle Emergence during Germination

The enzyme activity of βGlu was lower at initial stage of germination, peaked at about 72 h after imbibition (HAI), and then gradually decreased (Fig. 1). At 96 HAI, the radicles from amount of seeds were about to emerge, more than 90% of which emerged 24 h later. So, we assume that the endosperms of the majority seeds are rupturing at the 72 HAI and the radicles are emerging at 96 HAI. The seeds from the two time points together with 24 HAI seeds and dry seeds were prepared for transcriptome sequencing.

Transcriptome difference of the Seeds from the Four Developmental Stages

The amount of data from the transcriptome sequencing of four developmental seeds was between 3.5 and 4.0 Gb, with a total of 106,520 transcripts obtained. There were 72.61~91.43% clean reads that were mapped to the *N. tabacum* L. reference genome (Sierro *et al.,* 2014), and some of the data has been published (Li *et al.,* 2016). As shown in Table 1, the number of significant difference transcript (SDT) is closer among the three comparison groups. However, up-regulated SDT in Group 1 and 3 were significantly higher than that of Group 2. It is indicated that the seed metabolism might be more active during seed imbibition and radicle emergence.

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Table 1: The number of differential expressed transcript in the development of germinating tobacco seed

Groups	Differential analysis combination	Significant Difference	Significant Up-regulated number	Significant down-regulated number
I	Imbibed seeds/ Dry seeds	4261	2730	1531
II	Endosperm bursting seeds/ Imbibed seeds	4226	1969	2257
III	Radicle emerging seeds /Endosperm bursting seeds	4335	2708	1627

Table 2: The significant clustered model expression profiles of transcriptome STEM analysis

Nomber of model expression profiles	P-value	Gene Count	Expression pattern
29	4.00E-124	693	- ↑-
22	3.00E-93	506	-↓↑
45	1.00E-78	388	↑↓-
48	1.00E-46	645	↑-↑
23	6.00E-39	474	-↓-
31	1.00E-35	251	↑↓-
43	1.00E-24	201	↑↓↑
32	8.00E-20	219	↑↓↑
42	6.00E-17	221	↑↑↑
49	2.00E-16	575	↑↑↓
35	1.00E-13	191	↑↓-
46	2.00E-12	327	↑↓↑
21	3.00E-09	259	↓↑-

Time Series Analysis of Differential Expression Transcript in Four Developmental Stages of Germinating Seeds

STEM methods were used to cluster analysis of differential expression transcript from the four developmental germinating seeds. Thirteen patterns of the expression profiles were found to be significantly clustered (Fig. 2 and Table 2), including the transcript continuously up-regulated pattern (module 42), two stage up-regulated pattern (module 48), only one stage up-regulated pattern (module 29), and so on. It is worth noting that the continuously down-regulated and two stages of down-regulation pattern have not been found, and even one stage of down-regulated pattern clustered was just focused on the endosperm rupture. The results indicated that the vast majority of metabolisms were stimulated during seed imbibition and radicle emergence.

This study focuses on the transcript continuously up-regulated pattern, and this expression pattern included 221 genes. GO enrichment analysis found that these genes involved in signaling pathways are shown in Table 3, The results indicated that ABA-activated signal, cell wall hydrolysis, and light signal transduction metabolism were continuously enhanced during seed germination.

Hormone signaling pathways were induced during seed imbibition, including abscisic acid and ethylene activated signaling pathways, auxin transport and salicylic acid response (Table 4). The methionine metabolism was stimulated after imbibition, such as tryptophan decomposition, serine synthesis, and aspartic acid metabolism. Cell division, growth and differentiation were significantly increased, and cell wall hydrolytic enzyme activity increased significantly before endosperm rupture. Light signal pathway increased significantly in radicle emerging seeds, such as: red and far-red response, light

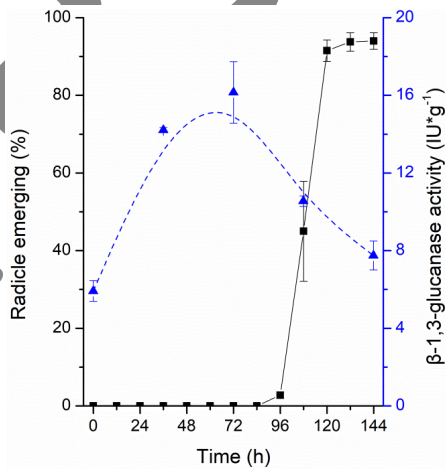

Fig. 1: Seed germination of tobacco and change of βGlu I activity

system II stability, light system I capture the light. The above results indicated that the metabolism of dried seeds gradually recovered in the process of water uptake, and the hormone activated signal played a key role in the start of seed germination; Cell wall hydrolysis and cell development were activated after imbibition pre the endosperm rupture; Photosynthesis was stimulated in the radicle emerged seeds.

Validation RNA-seq Results by RT-qPCR

To verify the accuracy of RNA-seq, 10 differentially expressed genes were randomly selected for RT-qPCR verification. The correlation coefficient between gene relative expression level and FPKM value was 0.769 (P =5.01E-03), and the results indicated that the gene expression information obtained by transcriptome sequencing was highly accurate (Fig. 3.)

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Table 3: The functional enrichment results of model expression profile number 42 from STEM analyses

GO ID	Functional categories	Query set	Refence set	P-value(*FDR*)
GO:0009789	positive regulation of abscisic acid-activated signaling pathway	4	69	0.012
GO:0019253	reductive pentose-phosphate cycle	4	60	0.00749
GO:0008422	beta-glucosidase activity	7	86	7.86E-06
GO:0005199	structural constituent of cell wall	4	27	0.000749
GO:0005546	phosphatidylinositol-4,5-bisphosphate binding	4	39	0.00245
GO:0004630	phospholipase D activity	4	47	0.00366
GO:0010114	response to red light	7	252	0.00301
GO:0009637	response to blue light	6	284	0.0268
GO:0010218	response to far red light	6	295	0.0314
GO:0015995	chlorophyll biosynthetic process	7	457	0.0473

Discussion

Seed germination is a complex biological process triggers with imbibition and ends with radicle emergence (Bewley, 1997). The development of embryo and the restriction of endosperm in the germinating seeds is an antagonistic event, and endosperm rupture is a precondition for the finish of germination in tobacco seeds. Class I β-1,3-glucanase (βGlu I) is a target enzyme for endosperm weakening that is accumulated after testa rupture but prior to endosperm rupture in tobacco seeds (Leubner-Metzger *et al.*, 1995). In this study, the activity of βGlu I peaks at about 72 HAI, 24 h later the radicle begins to emerge (Fig. 1). So we assume that the moment the endosperm of majority seeds is being ruptured. Cell elongation is requisite and is generally considered as sufficient for the completion of radicle emergence, cell division is not prerequisite (Kucera *et al.*, 2005). However, cell division, growth and differentiation metabolism were active before the endosperm rupture. It indicates that cell division is also important for radicle emerging, at least, which is prepared for the development of emerged radicle.

Methionine is the metabolic center in the control of germination, and its metabolites are extensively involved in biochemical pathways, which play a variety of roles in seed germination (Rajjou *et al.*, 2012). In this study, we found that few enzymes in methionine metabolism were stimulated in the geminating seeds. In *Arabidopsis* seed, enzymes in methionine pathway show different phases of expression during germination. Met synthase accumulates strongly prior to radicle emergence, whose level is not increasing with emerging or emerged radicle. However, AdoMet synthetase specifically accumulates at the time of radicle protrusion (Gallardo *et al.*, 2001, 2002a, 2002b). In this study, we also detected that the time point of methionine metabolism excited is pre radicular protrusion. For instance, serine anabolism and tryptophan catabolism are active at the moment. Serine is the precursor of cysteine and in turn it is the precursor of the antioxidant glutathione (GSH), which is involved in reactive oxygen species and nitric oxide metabolism to regulate seed germination (Bethke *et al.*, 2006; Bonsager *et al.*, 2010). Tryptophan is an important precursor for auxin biosynthesis in plants, which is the secondary hormone controlling seed dormancy under high concentration (Liu *et al.*, 2013; Li *et al.*, 2016).

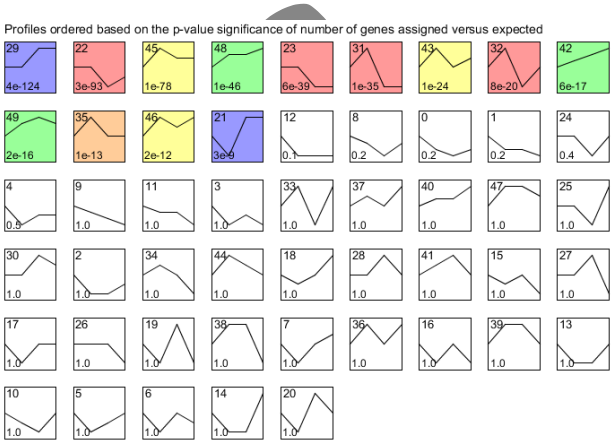

Fig. 2: STEM analysis of transcripts during tobacco seed germination

The horizontal axis shows the four germination phase, and the vertical axis denotes the transcript expression levels. Each module represents an expression pattern of time series in germinating seeds. The number in the upper left corner of module is the pattern number, and the lower left one is the p-value. In total, 13 patterns showed significant p-values ($p < 0.05$). In the case of 32, the line represents those transcripts that were up-regulated first during imbibition, then down-regulated with endosperm rupture, and then up-regulated in the radicle emerge

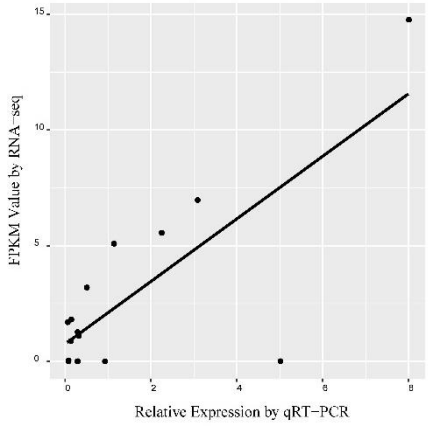

Fig. 3: The correlation between qRT-PCR and RNA-seq

It is well known that seed respiration is activated after a few min of imbibition (Weitbrecht *et al.*, 2011), however, whether photosynthesis can also perform during germination and when it starts is still poorly understood. In this study, signal pathways for photosynthesis were activated during

Table 4: Representative significant enriched GO terms of the differential expressed unigenes during seed germination

Seed status	GO ID	Go term	Refence set	Query	P-value (*FDR*)
Imbibition	GO:0009738	abscisic acid-activated signaling pathway	38	547	4.50E-05
	GO:0009873	ethylene-activated signaling pathway	28	353	0.00011
	GO:0010540	basipetal auxin transport	11	84	0.00172
	GO:0009751	response to salicylic acid	19	256	0.00634
	GO:0051740	ethylene binding	8	36	0.000599
Endosperm burst	GO:0006569	tryptophan catabolic process	14	142	0.000104
	GO:0009800	cinnamic acid biosynthetic process	5	16	0.000614
	GO:0019344	cysteine biosynthetic process	26	511	0.00195
	GO:0042398	cellular modified amino acid biosynthetic process	8	78	0.00654
	GO:0045330	aspartyl esterase activity	14	100	1.73E-06
	GO:0045548	phenylalanine ammonia-lyase activity	5	15	0.000458
	GO:0044318	L-aspartate:fumarate oxidoreductase activity	5	19	0.00138
	GO:0008734	L-aspartate oxidase activity	5	20	0.00171
	GO:0004185	serine-type carboxypeptidase activity	10	113	0.00413
	GO:0008234	cysteine-type peptidase activity	13	198	0.00836
	GO:0007076	mitotic chromosome condensation	13	23	9.59E-09
	GO:0010389	regulation of G2/M transition of mitotic cell cycle	41	229	1.08E-08
	GO:0042127	regulation of cell proliferation	26	146	1.08E-08
	GO:0008283	cell proliferation	106	567	1.14E-08
	GO:0010075	regulation of meristem growth	39	484	1.22E-08
	GO:0045490	pectin catabolic process	14	94	8.19E-07
	GO:0009932	cell tip growth	17	193	4.54E-05
	GO:0048767	root hair elongation	31	546	4.54E-05
	GO:0010015	root morphogenesis	8	38	5.57E-05
	GO:0008361	regulation of cell size	12	98	5.76E-05
	GO:0009793	embryo development ending in seed dormancy	20	2201	0.000634
	GO:0042545	cell wall modification	15	196	0.000791
	GO:0009830	cell wall modification involved in abscission	3	4	0.00138
	GO:0009558	embryo sac cellularization	6	31	0.00148
	GO:0009825	multidimensional cell growth	18	285	0.00149
	GO:0008422	beta-glucosidase activity	19	86	1.08E-08
	GO:0030599	pectinesterase activity	23	145	1.08E-08
	GO:0016985	mannan endo-1,4-beta-mannosidase activity	9	22	2.76E-08
	GO:0009044	xylan 1,4-beta-xylosidase activity	9	29	4.09E-07
	GO:0016762	xyloglucan:xyloglucosyl transferase activity	12	67	1.04E-06
	GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	24	293	1.42E-06
	GO:0042973	glucan endo-1,3-beta-D-glucosidase activity	10	83	0.000445
Radicle emerge	GO:0009765	photosynthesis, light harvesting	23	80	2.39E-08
	GO:0009773	photosynthetic electron transport in photosystem I	19	130	5.40E-07
	GO:0010207	photosystem II assembly	39	496	1.07E-06
	GO:0010114	response to red light	25	252	5.56E-06
	GO:0042549	photosystem II stabilization	5	8	8.87E-05
	GO:0009637	response to blue light	23	284	0.000507
	GO:0019684	photosynthesis, light reaction	16	154	0.000599
	GO:0010218	response to far red light	23	295	0.000863
	GO:0009768	photosynthesis, light harvesting in photosystem I	5	12	0.000938
	GO:0016168	chlorophyll binding	27	99	2.39E-08

radicle emergence, indicated that the photosynthesis began at the later stage of seed germination, or at least illustrated the signal pathways for seedling photosynthesis were activated at the germination stage. Seeds of *Posidonia oceanica*, a kind of sea grasses, display activity of photosynthesis during germination, which subsequently enhanced seedling growth in leaves and roots (Celdran and Marin, 2013). It's an interesting scientific question but few studied about whether the seeds are photosynthetic during germination. In development of *A.thaliana* seeds, photosynthesis is vigorous in the seed mature stage (Eastmond *et al.*, 1996), and inhibition of photosynthesis in the developmental embryo is harmful for seed storability and germination timing (Allorent *et al.*, 2015).

Conclusion

Dry seeds, imbibed seeds, endosperm rupturing seeds, radicle emerging seeds were comparatively studied for their differences in transcriptomes. The number of up-regulated SDT during seed imbibition and radicle emergence were more than that of endosperm rupture. STEM program analysis showed that thirteen patterns of the expression profiles were found to be significantly clustered, including the continuously, two stages and only one stage up-regulated pattern, but not continuously or two stages of down-regulated pattern. Of them, continuously up-regulated pattern was focuses on and it included 221 genes. GO enrichment analysis found that ABA signal, cell wall

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hydrolysis, and light signal transduction metabolism were continuously enhanced during seed germination. In addition, hormone signal played a key role in the initiation of seed germination; Cell wall hydrolysis and cell development were activated pre endosperm rupture; photosynthesis has been stimulated in the radicle emerging seeds.

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