Identification of brassinosteroid responsive genes in *Arabidopsis* by cDNA array

HU Yuxin (胡玉欣)[1], WANG Zhengke (汪政科)[2], WANG Yonghong (王永红)[1], BAO Fang (包 方)[1], LI Ning (李 凝)[3], PENG Zhenhua (彭镇华)[2] & LI Jiayang (李家洋)[1]

1. Institute of Genetics, Chinese Academy of Sciences, Beijing 100101, China;
2. Institute of Forest, Chinese Academy of Forestry, Beijing 100091, China;
3. Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

Correspondence should be addressed to Li Jiayang (email: jyli@genetics.ac.cn)

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Abstract We have systematically monitored brassinosteroid (BR) responsive genes in a BR-deficient mutant *det2* suspension culture of *Arabidopsis* by using a cDNA array approach. Among 13000 cDNA clones arrayed on filters, 53 BR responsive clones were identified and designated *BRR1—BRR53*. Sequence analysis of 43 clones showed that 19 clones are novel genes, 3 clones are genes involved in the control of cell division, 4 clones are genes related to plant stress responses, 4 clones are transcriptional factor or signal transduction component genes, and 3 clones are genes involved in RNA splicing or structure forming. In addition, we also found that BR regulated the transcription of genes related to many physiological processes, such as photoreaction, ion transportation and some metabolic processes. These findings present molecular evidence that BR plays an essential role in plant growth and development.

Keywords: cDNA array, brassinosteroid responsive gene (BRR), *Arabidopsis thaliana*.

Brassinosteroids (BRs) are the steroid hormones found in plants[1]. Previous studies have shown that BR, like other plant hormones such as auxin and cytokinin, is the hormone essential for normal plant growth and development. BR regulates plant cell elongation and division, vascular differentiation, senescence, pollen fertility and stress responses[2,3]. Recent studies on BR deficient mutant have elucidated the biosynthesis pathway of BR in higher plants[3,4]. However, the research on its molecular mechanism and signal transduction is less fruitful, at least in part because little has been known about its regulated genes. Although a few BR-regulated genes were identified recently, the molecular mechanism of BR action remains unclear. Recently, an effective technique called cDNA microarray has been developed and used to monitor gene expression in plants[5,6]. However, its application to identification of BR-regulated genes has not been reported so far.

To identify BR responsive genes, we have developed a simple but effective cDNA array method to monitor gene expression based on filter hybridization[7]. With this method, BR responsive genes in *Arabidopsis thaliana* were identified and analyzed among 13000 arrayed cDNA

clones and a part of BR responsive genes have been cloned. This paved a way for further investigation and understanding of mechanism of BR function in plant growth and development.

1 Materials and methods

1.1 Plant material

Arabidopsis thaliana BR-deficient mutant *det2*[8] was used to produce cultured suspension cells.

1.2 Suspension culture and hormone treatment

Seeds of *det2* were soaked in 70% ethanol for 3—5 min, and then in 10% Bleach for 10—15 min for surface sterilization, washed 3—4 times with sterilized water. For callus induction, the sterilized seeds were cultured on B5 medium containing 2% glucose, 4.5 **ì mol/L 2,4-dichlorophenoxyacetic** acid (2,4-D), 0.45 **ì mol/L** kinetin (KT) and 0.8% agar in the dark at 25℃. The suspension culture was established by suspending well-grown calli in the liquid medium as described above and maintained in constant low light with orbital shaking at 130 rpm. The medium was replaced weekly.

Before being treated with 24-epi-brassinolide (BL), suspension cultures were washed three times with B5 medium and maintained in B5 medium for 48 h for hormone starvation. Suspension cultures were treated for 2 h with 5 **ì mol/L BL or equal volume of DMSO (control), respectively.** RNA was extracted and used for preparation of cDNA array probe. For Northern blot analysis, suspension cultures were treated with 5 **ì mol/L BL for 0, 1, 2 and 4 h.**

1.3 cDNA array

The cDNA array procedures were basically carried out as previously described[7]. The high density filters were prepared using the Biomek 2000 HDRT system, and were probed with α-^{32}P-dCTP-labelled first strand cDNA. The signal was analyzed with a phosphoimager (Molecular Dynamics, CA, USA).

1.4 RNA extraction and RNA gel-blot analysis

Total RNA was isolated according to the guanidinium-thiocyanate-chloroform extraction procedure[9]. Suspension cells were powdered in liquid nitrogen, extracted with guanidinium thiocyanate-phenol-chloroform, precipitated by ethanol, purified with LiCl and chloroform each time. Total RNA was suspended in nuclease-free water and stored at −20℃ after quantification.

The total RNA of 15 μg of each sample was fractionated in a formaldehyde agrose gel, blotted onto nylon filters and immobilized in a vacuum baker at 80℃ for 2 h. Hybridization was performed in Church buffer[10] at 65℃ for 16—20 h and followed by washing of the filter with mild stringency.

1.5 Sequence analysis

Differentially expressed cDNA clones were subcloned into a pBluescript II SK(+) vector and

sequenced with ABI373A DNA Sequencer (Perkin Elmer, USA). DNA or protein homology search against GeneBank database was performed using the program Blast.

2 Results

2.1 Identification of BR responsive clones

To understand the molecular mechanism of BR function, a cDNA array approach was used to identify BR responsive genes. A set of 12 high density filters containing triplicate 13000 cDNA clones were prepared with Biomek 2000 and BR responsive clones were identified with *det2* suspension culture of *Arabidopsis.* The *det2* mutant was chosen because it is defective in an early step of the BR biosynthetic pathway and has very low level of endogenous BR[8]. Fig. 1 shows the gene expression patterns of *det2* cells in one of the filters. Although the expression pattern was similar, there were some clones whose expression levels were distinctly different between the BL-treated and the control. Some were induced by BL, while some were suppressed (fig. 1). Out of the 13000 arrayed clones, 53 were found to be BR responsive and designated *BRR1—BRR53.*

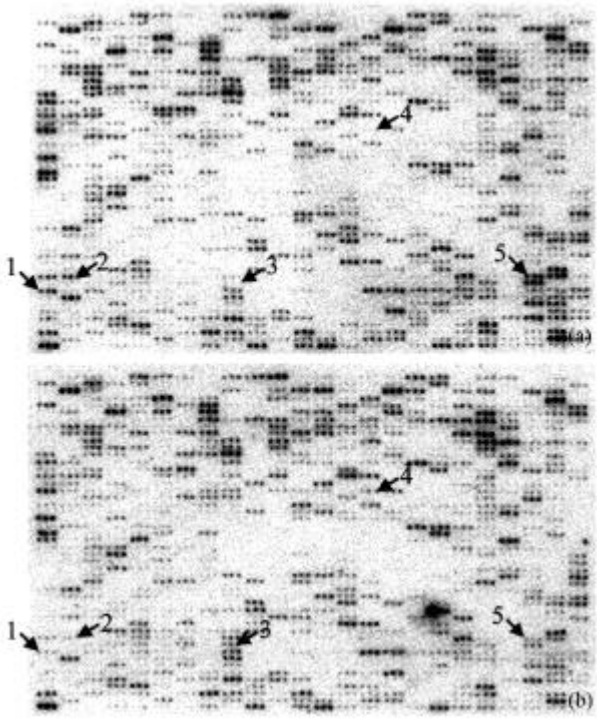

Fig. 1. Gene expression patterns of *det2* suspension cells treated with BL. (a) Treatment with 5 μmol/L BL for 2 h; (b) the control. Arrows indicate the differentially expressed clones: 1, 2 and 5 are BL-induced clones; 3 and 4 are BL-suppressed clones.

To confirm the BR responsive clones identified, RNA gel-blot analysis was performed with 25 clones and the results of 10 clones were shown in fig. 2. Most of these clones demonstrated the same responsiveness as those shown in the cDNA array. However, the Northern results of seven clones (*BRR19, BRR25, BRR2, BRR9, BRR33, BRR35, BRR43*) were not well reproducible in the Northern blot analyses (see below).

2.2 BR responsive genes

A total of 43 differentially expressed cDNA clones were subcloned into a pBluescript II SK (+) vector. Sequencing and homology analyses revealed that 19 clones have their corresponding genomic DNA sequence stored in GeneBank, whereas the other 24 clones have homologous cDNAs or protein sequences instead. BR responsive genes were classified according to their functions as shown in table 1.

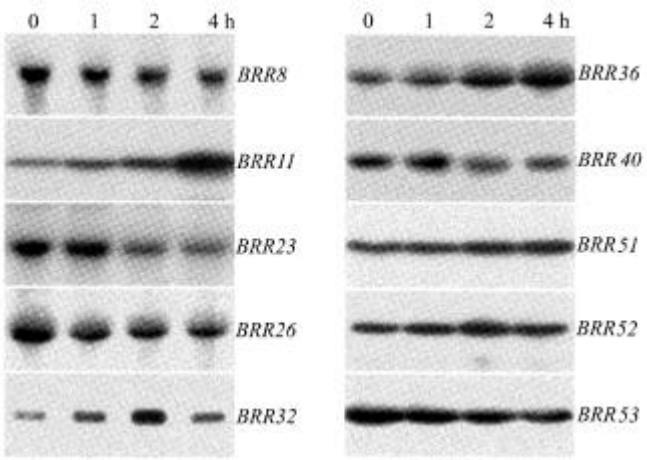

Fig. 2. Northern hybridization of some *BRR* clones. RNA was isolated from *det2* cells treated with 5 μmol/L BL for 0, 1, 2 and 4 h. Total RNA of 15 μg was loaded in each lane.

As shown in table 1, BR responsive genes are involved in many aspects of plant growth and development. Three genes are related to cell division or differentiation control, including a D-type cyclin gene *CycD3*, which proves that BR is involved in cell division. Four genes are involved in plant stress responses, including two heat-shock protein genes and a cyclophilin gene. In addition four genes are involved in transcriptional regulation or signal transduction of BR, including three transcription factors and a putative protein kinase gene. Another three genes are related to RNA splicing and structure. BR was found to play a role in plant photoreaction, ion transportation and other metabolic processes. Sequencing analysis also demonstrated that the unreproducible genes in Northern blot analysis were genes mainly related to photosynthesis (see section 3).

3 Discussion

Differential gene expression is associated with the plant growth and development and physiological responses to the internal/external environment cues. The plant hormones play many roles in growth and development as well as all metabolic processes including affecting tissue- and stage-specific gene expression. In order to understand the mechanism of their actions, we have systematically studied the BR responsive genes by cDNA array. Furthermore, because we employed the BR-deficient mutant cell line as the material to deprive the interference of the endogenous BR to some extent, it is possible that the BR-responsive genes that we identified may have the genes that are difficult to be found in normal plants. Moreover, the identification of these BR responsive genes or the BR-regulatory genes paves a way for further study of the BR signal transduction and regulatory mechanism.

Three genes involved in controlling of cell division were found to be BR responsive. Among them, CycD3 is a D-type cyclin which functions as a mediator of environmental stimuli to drive cell division[11], and the activation of cell division by cytokinin has been proved to be through the transcriptional induction of *CycD3*[12]. Previously we also presented the evidence that the promo-

tive effect of BR on cell division involves a distinct *CycD3*-dependent pathway[13]. Although the *in vivo* function of Myb-like protein remains unknown, it has been suggested to be involved in differentiation or other cellular process[14]. *MLO2* is another gene related to cell division and found in yeast. Its overexpression leads to the suppression of yeast cell division[15].

Table 1 Genes (clones) responsive to BR treatment

Function	Clone	+/–[a)]	Homologous gene/protein	Identity (D/P[b)])	Accession number
Unknown	19	+/–			
Related cell division	*BRR36*	+	CycD3	D97	X83371
	BRR53	–	Myb-like protein	D91	P34127
	BRR21	–	MLO2(Yeast)	P43	T40419
Related to stress	*BRR32*	+	Cyclophilin	D93	U40399
response	*BRR11*	+	Hsp90A(81)	D93	Y07613
	BRR52	+	Hsp90B(82)	D98	Y11827
	BRR6	+	Putative stress protein	P40	CAB88296
Related to signal	*BRR8*	–	RAV1 DNA binding protein	D92	AB013886
transduction	*BRR23*	–	CCAAT binding factor	P45	NM031553
	BRR26	–	Putative kinase(Yeast)	P45	AJ303007
	BRR27	–	GAGA transcription factor	P47	L22205
Related to RNA	*BRR22*	–	RNA helicase	D98	X98130
	BRR40	–	U2snRNP auxiliary factor	P74	AJ291762
	BRR51	–	PolyA binding protein	D97	L19418
Others	*BRR1*	–	Ferritin	D92	AF326869
	BRR3	+	CHLH(Mg^{2+} chelatase)	D98	Z68495
	BRR14	+	PEALI 4 mRNA	D85	L43081
	BRR25	?[c)]	*Ats1A*	D85	AF360124
	BRR19	?	*Ats1B*	D93	P10796
	BRR9	?	*Ats1A*	D91	AF360124
	BRR43	?	oxygen-evolving complex protein 3(photosystem II)	D99	AF026400
	BRR49	+	1-acyl-sn-glycerol-3-phosphote acyltransferase	D88	Z95637
	BRR13	+	Prolylcarboxypeptidase	P69	AAF18628
	BRR2	?	ATP synthase delta subunit	D83	AAK05865

a) +, genes induced by BL treatment; –, genes suppressed by BL treatment. b) D, DNA; P, protein. c) ?, BR responsive clones that were not well reproducible in Northern blot analyses.

BR can also enhance plant stress tolerance, especially to heat, chilling and salt[3]. We found that 4 genes involved in stress response are BR-inducible. The finding that two members of *Hsp90* gene family, *Hsp81* and *Hsp82*, were induced by BL suggests that BR may enhance the plant heat tolerance by regulating the expression of *Hsp90*[16]. In human and animals, the intracellular receptors of steroid hormones, such as estrogen, progestin, androgen and glucocorticoid, are large complexes. Not only is Hsp90 a component of the complex that makes steroid receptor maintain its active conformation, but it is also involved in the signal transduction of this steroid hormone[17].

Cyclophilin, another conserved molecular chaperone that normally exists in animal and plant cells, plays a role in protein folding. Seven *cyclophilin* genes have been found in *Arabidopsis thaliana*[18]. Yeast cyclophilin-deficient mutant ceases to grow under heat shock, but grow well under the normal condition[19]. The *cyclophilin* was also found to be inducible upon heat shock in

maize and soybean[20]. These results suggest that cyclophilin is an important protein involved in plant stress tolerance.

We also found that BR regulates the transcription of 4 genes related to BR signal transduction or transcriptional and post-transcriptional regulation. RAV1 is a DNA binding protein with two DNA binding domains whose cellular functions remain unknown[21]. BRR26, a putative protein kinase, contains a highly conserved protein kinase domain, indicating that this protein kinase is involved in BR signal transduction. The finding that BL regulates CCAAT binding factor and GATA transcription factor suggests that BR regulates expression of some of the genes through transcription factor. Moreover, transcriptional alterations of RNA helicase, U2 snRNP auxiliary factor and polyA binding protein imply that BR regulates the gene expression either at the post-transcriptional or at the translational level.

BRs have also been shown to play a role in photoreaction[22] and several photosynthesis-related genes, such as *Ats,* have been shown to have an altered gene expression in the BR-deficient mutant[23]. In our experiments, we also observed that the transcription of four photosynthesis-related genes are affected by the BR treatment. However, they were not well reproducible in Northern blot analysis. It may be due to the materials that we used were cell cultures, in which photoreaction is known to be unessential for the growth and proliferation. Mg^{2+} chelatase, a key enzyme that chelates Mg^{2+} to porphyrin in chlorophyll biosynthesis, is composed of three subunits, CHLI, CHLD and CHLH[24]. Induction of the *CHLH* by BR shows that BR is involved in the photoreaction regulation. The identification of BR responsive genes also suggests that BR regulates ion transportation and many other metabolic processes (metabolic enzyme genes, ATP synthase gene as shown in table 1).

Although the functions of many BR-responsive genes are still not clear, the investigation of these novel BR-related genes will greatly broaden our knowledge on the molecular mechanism of BR action in higher plants.

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References

1. Grove, M. D., Spencer, G. F., Rohwedder, W. K. et al., Brassinolide, a plant growth-promoting steroid isolated from Brassica napus pollen, Nature, 1979, 281: 216—217.
2. Mandava, N. B., Plant growth-promoting brassinosteroids, Annu. Rev. Plant Physiol. Plant Mol. Biol., 1988, 39: 23—52.
3. Clouse, S. D., Sasse, J. M., Brassinosteroids: essential regulators of plant growth and development, Annu. Rev. Plant Physiol. Plant Mol. Biol., 1998, 49: 427—451.
4. Altmann, T., Recent advances in brassinosteroid molecular genetics, Curr. Opin. Plant Biol., 1998, 1: 378—383.
5. Aharoni, A., Keizer, L. C. P., Bouwmeester, H. J. et al., Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarray, Plant Cell, 2000, 12: 647—661.
6. Reymond, P., Weber, H., Damond, M. et al., Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis, Plant Cell, 2000, 12: 707—719.

7. Hu, Y., Han, C., Mou, Z. et al., Monitoring gene expression by cDNA array, Chin. Sci. Bull., 1999, 44: 441—444.
8. Fujioka, S., Li, J., Choi, Y. H. et al., The Arabidopsis *deetiolated2* mutant is blocked early in brassinosteroid biosynthesis, Plant Cell, 1997, 9: 1951—1962.
9. Wadsworth, G. J., Redinbaugh, M. G., Scandalios, J. G., A procedure for small-scale isolation of plant RNA suitable for RNA blot analysis, Anal. Biochem., 1988, 172: 279—283.
10. Church, G. M., Gilbert, W., Genomic sequencing, Proc. Natl. Acad. Sci. USA, 1984, 81: 1991—1995.
11. Huntley, R. P., Murray, J. A. H., The plant cell cycle, Curr. Opin. Plant Biol., 1999, 2: 440—446.
12. Riou-Khamlichi, C., Huntley, R., Jacqmard, A. et al., Cytokinin activation of Arabidopsis cell division through a D-type cyclin, Science, 1999, 283: 1541—1544.
13. Hu, Y., Bao, F., Li, J., Promotive effect of brassinosteroids on cell division involves a distinct *CycD3*-induction pathway, Plant J., 2000, 24: 693—701.
14. Hirayama, T., Shinozaki, K., A cdc5+ homolog of a higher plant, Arabidopsis thaliana, Proc. Natl. Acad. Sci. USA, 1996, 93: 13371—13376.
15. Javerzat, J. P., Cranston, G., Allshire, R. C., Fission yeast genes which disrupt mitotic chromosome segregation when overexpressed, Nucl. Acids Res., 1996, 24: 4676—4683.
16. Dhaubhadel, S., Chaudhary, S., Dobinson, K. F. et al., Treatment with 24-epibrassinolide, a brassinosteroid, increases the basic thermotolerance of *Brassica napus* and tomato seedlings, Plant Mol. Biol., 1999, 40: 333—342.
17. Beato, M., Herrlich, P., Schytz, G., Steroid hormone receptors: many actors in search of a plot, Cell, 1995, 83: 153—156.
18. Chou, I. T., Gasser, C. S., Characterization of the cyclophilin gene family of *Arabidopsis thaliana* and physiologenetic analysis of known cyclophilin proteins, Plant Mol. Biol., 1997, 35: 873—892.
19. Sykes, K., Gething, M. J., Sambrook, J., Proline isomerases function during heat shock, Proc. Natl. Acad. Sci. USA, 1993, 90: 5853—5857.
20. Marivet, J., Frendo, P., Burkard, G., DNA sequence analysis of a cyclophilin gene from maize: developmental expression and regulation by salicylic acid, Mol. Gen. Genet., 1995, 247: 222—228.
21. Kagaya, Y., Ohmiya, K., Hattori, T., RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plant, Nucleic Acids Res., 1999, 27: 470—478.
22. Neff, M. M., Nguyen, S. M., Malancharuvil, E. J. et al., *BAS1*: a gene regulating brassinosteroid levels and light responsiveness in Arabidopsis, Proc. Natl. Acad. Sci. USA, 1999, 96: 15316—15323.
23. Chory, J., Nagpal, P., Peto, C. A., Phenotypic and genetic analysis of *det2*, a new mutant that affects light-regulated seedling development in Arabidopsis, Plant Cell, 1991, 3: 445—459.
24. Papenbrock, J., Grafe, S., Kruse, E. et al., Mg^{2+}-chelatase of tobacco: identification of a *ChlD* cDNA sequence encoding a third subunit, analysis of the interaction of the three subunits with the yeast two-hybrid system, and reconstitution of the enzyme activity by co-expression of recombinant ChlD, ChlH and ChlI, Plant J., 1997, 12: 981—990.

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作者： 胡玉欣， 汪政科， 王永红， 包方， 李凝， 彭镇华， 李家洋
作者单位： 胡玉欣,王永红,包方,李家洋(Institute of Genetics, Chinese Academy of Sciences, Beijing 100101, China)， 汪政科,彭镇华(Institute of Forest, Chinese Academy of Forestry, Beijing 100091, China)， 李凝(Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China)
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参考文献(24条)

1. Sykes K;Gething M J;Sambrook J Proline isomerases function during heat shock[外文期刊] 1993

2. Wadsworth G J;Redinbaugh M G;Scandalios J G A procedure for small-scale isolation of plant RNA suitable for RNA blot analysis 1988

3. Kagaya Y;Ohmiya, K;Hattori, T RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plant[外文期刊] 1999(2)

4. Marivet J;Frendo.P;Burkard.G DNA sequence analysis of a cyclophilin gene from maize: developmental expression and regulation by salicylic acid 1995

5. Grove M D;Spencer, G. F;Rohwedder, W. K Brassinolide, a plant growth-promoting steroid isolated from Brassica napus pollen 1979

6. Fujioka S;Li, J;Choi, Y. H The Arabidopsis deetiolated2 mutant is blocked early in brassinosteroid biosynthesis[外文期刊] 1997

7. Hu y;Han, C;Mou, Z Monitoring gene expression by cDNA array[外文期刊] 1999(5)

8. Reymond P;Weber, H;Damond, M Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis[外文期刊] 2000

9. Aharoni A;Keizer, L. C. P;Bouwmeester, H. J Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarray 2000

10. Altmann T Recent advances in brassinosteroid molecular genetics,Curr[外文期刊] 1998

11. Clouse S D;Sasse, J. M Brassinosteroids: essential regulators of plant growth and development, Annu. Rev. Plant Physiol[外文期刊] 1998(0)

12. Mandava N B Plant growth-promoting brassinosteroids, Annu. Rev. Plant Physiol[外文期刊] 1988

13. Papenbrock J;Grafe, S;Kruse, E Mg2+-chelatase of tobacco: identification of a ChlD cDNA sequence encoding a third subunit, analysis of the interaction of the three subunits with the yeast two-hybrid system, and reconstitution of the enzyme activity by co-expression of recombinant ChlD 1997

14. Chory J;Nagpal, P;Peto, C. A Phenotypic and genetic analysis of det2, a new mutant that affects light-regulated seedling development in Arabidopsis 1991

15. Neff M M;Nguyen.S.M;Malancharuvil.E.J BAS1: a gene regulating brassinosteroid levels and light

responsiveness in Arabidopsis[外文期刊] 1999(26)

16. Chou I T;Gasser, C. S Characterization of the cyclophilin gene family of Arabidopsis thaliana and physiologenetic analysis of known cyclophilin proteins[外文期刊] 1997(6)

17. Beato M;Herrlich, P;Schytz, G Steroid hormone receptors: many actors in search of a plot[外文期刊] 1995

18. Dhaubhadel S;Chaudhary, S;Dobinson, K. F Treatment with 24-epibrassinolide, a brassinosteroid, increases the basic thermotolerance of Brassica napus and tomato seedlings[外文期刊] 1999

19. Javerzat J P;Cranston, G;Allshire, R. C Fission yeast genes which disrupt mitotic chromosome segregation when overexpressed[外文期刊] 1996

20. Hirayama T;Shinozaki·K A cdc5+ homolog of a higher plant, Arabidopsis thaliana 1996

21. Hu y;Bao, F;Li, J Promotive effect of brassinosteroids on cell division involves a distinct CycD3-induction pathway[外文期刊] 2000(5)

22. Riou-Khamlichi C;Huntley, R;Jacqmard, A Cytokinin activation of Arabidopsis cell division through a D-type cyclin[外文期刊] 1999

23. Huntley R P;Murray, J. A. H The plant cell cycle[外文期刊] 1999(6)

24. Church G M;Gilbert, W Genomic sequencing[外文期刊] 1984

引证文献(3条)

1. Guang-Zuo Luo. Yong-Jun Wang. Zong-Ming Xie. Jun-Yi Gai. Jin-Song Zhang. Shou-Yi Chen The Putative Ser/Thr Protein Kinase Gene GmAAPK from Soybean is Regulated by Abiotic Stress[期刊论文]-植物学报（英文版） 2006(3)

2. 何新建. 陈建权. 张志刚. 张劲松. 陈受宜 Identification of salt-stress responsive genes in rice (Oryza sativa L.) by cDNA array[期刊论文]-中国科学C辑(英文版) 2002(5)

3. BAO Fang. HU Yuxin. LI Jiayang Identification of auxin responsive genes in Arabidopsis by cDNA array[期刊论文]-科学通报(英文版) 2002(7)