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CLONING AND CHARACTERIZATION OF PEROXIDASE GENE IN *PHALAENOPSIS*

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Abstract

Plant peroxidases oxidize phenolic substrates at the expense of H_2O_2 perform a significant function in responses to environmental stresses *via* the regulation of H_2O_2 in plants. In this study, a full-length cDNA encoding a peroxidase was cloned and sequenced from leaf explants of *Phalaenopsis* by RT-PCR and RACE methods. The cDNA designated as *PhPOD* (GenBank accession No. FJ161978), is 1251 bp and contains a 1041 bp open reading frame (ORF), which encodes a 347 amino acid peroxidase precursor, with a 24 aa N-terminal signal peptide targeting to ndoplasmic reticulum. The putative protein has a calculated molecular weight of 37.22 kDa and a calculated pI of 7.55. Sequence analysis showed that the deduced amino acid sequence of *PhPOD* shares high identity with the reported POD protein sequences in database and is a typical Class III of POD family in plants. PhPOD possesses all active residues and two Ca^{2+} -binging sites present in peroxidase isoenzymes C, as well as six N-glycosylation sites. Semi-quantitative RT-PCR revealed that the expression of *PhPOD* was increased just before explant browning, and decreased with the aggravating of explants browning. These results indicate a possible function of PhPOD and provide an alternative way for controlling explant browning in tissue culture.

Introduction

Explant browning is one of the most common problems in the early stages of plant tissue culture. It can be classified as either an enzymatic or non-enzymatic process. In enzymatic browning, peroxidase (POD, EC 1.11.1.7), polyphenol oxidase (PPO EC 1.10.3.1or EC 1.14.18.1), and phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) are involved (Bhat & Chandel, 1991; Ye *et al*., 2004). The substrates of POD and PPO enzymes are mainly polyphenolic compounds. Inhibition of the PPO and POD activities delayed litchi pericarp browning (Jiang *et al*., 2004). POD is involved in enzymatic browning in fruits and vegetables (Zhang *et al*., 2005; Chisari *et al*., 2007; Yingsanga *et al*., 2008). There was a correlation between POD activity and cellular browning in the browned pericarp of harvested litchi fruit in which higher POD activity was observed (Critchley *et al*., 1995; Sarni-Manchado *et al.,* 2000). Laukkane *et al*., (1999) found that the high POD activities caused rapidly and early browning in scots pine callus tissues. Our previous report demonstrated that with the browning of explants, the activities of PAL , PPO and POD were increased and *PAL* gene expression was up-regulated after 3 days of tissue culture (Xu & Li, 2006; Xu *et al*., 2007). POD activity increased before leaf explant browning and its isoenzyme pattern changed during browning *Phalaenopsis* explant (Xu *et al*., 2006). Tang *et al*., (2004) investigated the influence of antioxidants, polyvinylpoly- pyrrolidone (PVPP) and 1,4-DL-threitol (DTT) on regeneration of Virginia pine (*Pinus virginiana* Mill.) *In vitro*. The results showed that the addition of antioxidants reduces and inhibits browning by reducing the accumulation of peroxidase .

Peroxidases (POD, EC 1.11.1.7) are a ubiquitous class of enzymes that oxidize a vast array of organic and inorganic reducing compounds in the presence of hydrogen peroxide. Plant peroxidases are heme-containing glycoproteins designated as class III peroxidases on the basis of various conserved domains to distinguish them from class I ascorbate peroxidases (also found in plants) and class II fungal peroxidases (Stephanie *et al*., 2005). All class III peroxidases possess a signal peptide that targets the proteins for secretion *via* the endoplasmic reticulum.

Some plant peroxidase genes are upregulated or down-regulated under certain environmental conditions, such as various forms of stress, wounding, or pathogen attack (Cheong *et al*., 2002; Do *et al*., 2003; Razem *et al*., 2003; Almagro *et al*., 2009). Transgenic tobacco (*Nicotiana tabacum*) plants overexpressing the sweet potato peroxidase (*SWPA4*) gene exhibited higher POD specific activity and significantly enhanced tolerance to a variety of abiotic and biotic stresses (Kim *et al*., 2008). Wounding induced H_2O_2 accumulation, and showed a high level of peroxidase activity in birch leaves wound sites (Ruuhola *et al.*, 2006). When explants were cut from the plant, the reactive oxygen bursts in response to wounding (Razem *et al*., 2003; Musetti *et al*., 2005), which damaged the integrality of cell membrane and caused the tissue browning (Dan 2008; Lin *et al*., 2005). However, the roles for peroxidase during browning of the explant remain unknown.

It was reported in previous studies that a PPO homological gene were cloned from *Phalaenopsis* (Xu *et al*., 2009). In the present paper, we describe the molecular cloning of a full-length of a class III peroxidase gene, *PhPOD*, from *Phalaenopsis* by RT-PCR and RACE methods. Its expression in leaf explant during browning was analyzed by semi-quantitative RT-PCR to investigate the role of POD in enzymatic browning.

Materials and Methods

Plant material and culture conditions: Leaves of *Phalaenopsis* (*Doritaenopsis Queen Bee* "Red Sky") plants, were cut into 0.5 cm × 0.5cm segments and cultured on Murashige & Skoog medium (Murashige & Skoog, 1962) solidified with 0.8% (w/v) agar at pH 5.8, supplied with 3mg/L 6-BA, All cultures were maintained under cool white fluorescent light (approximately 35 μmol m^{-2} s^{-1} photon flux density and with a 16 h photoperiod) at 24±2ºC.

RNA extraction and RT-PCR amplification of the

conserved region: Total RNA were extracted from leaf explants at 0, 6, 12, 24, 72, 144 and 288 hours after culture with Trizol Reagent (Invitrogen, USA). Two degenerated primers PhPOD1 and PhPOD2 (Table 1) were designed according to the conserved nucleotides and amino acid sequences of POD (Smith & Veith, 1998; Welinder *et al*., 2002; Wu *et al*., 2006) An RT-PCR were performed with these two primers. The RT-PCR procedure started with 30 min reverse transcription at 50ºC and denatured at 94ºC for 2 min, followed by 34 cycles of amplification (94ºC for 30 s, 55ºC for 45 s, 72ºC for 1 min).

Table 1. Primers used in the cloning of the full-length cDNA of *PhPOD* by RACE.

Primer	Primer sequence (5'-3')
Oligo dT	5'- TTT TTT TTT TTT TTT TTT TTT TTT TN$_{-1}$N-3'
POD1	5'-ATGGATCAAACNTTCTTGAATCCTAA-3' (N=G,C,AorT)
POD2	5'-AGTTNGCNCGTATCTCNCCTTGATTNCC-3'
POD3	5'-AACCGCATCTTCCCTTCTCGTGA-3'
POD4	5'-AGCAGTTTCTCCTTCTTCATCCACA-3'
POD3-1	5'- ATGGTGAAGATGGGGCAGCTGAG-3'
POD3-2	5'-AGCTGAGCGTGCTGACTGGTGGAAA-3'
POD5-1	5'-AGCACAGTCGTGTTGGCGGAGTT-3'
POD5-2	5'-AAGGTTTGGTCCATGGTAGGGTCAC-3'
5'Race outer	5'-CATGGCTACATGCTGACAGCCTA-3'
5'Race inner	5'-CGCGGATCCACAGCCTACTGATGATCAGTCGATG-3'
3'Race 1	5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTT TT-3'
3'Race 2	5'-GACTCGAGTCGACATCG-3'

RACE PCR amplification for *PhPOD* 5'- and 3'-ends: Based on the sequence of the cloned conserved fragment, primers PhPOD3-1 and PhPOD3-2 were designed, synthesized and used for the following PCR. The first round PCR was performed with primer PhPOD3-1 and 3'Race1. The PCR product was diluted 50-fold and 1 µl of it was used as the template for the nested PCR with primers of PhPOD3-2 and 3'Race2. The 5'cDNA ends were obtained with TaKaRa RACE cDNA Amplification Kit (TaKaRa, Japan). For 5'-RACE, 1 µg total RNA was reversely transcribed with the 5'-RACE outer Primer (provided in the kit) and 5'-Race primer. Based on the sequence of the 3'-RACE product, the specific primers PhPOD5-1, PhPOD5-2 and PhPOD5-3 were designed and synthesized. The first round of PCR was performed with primer PhPOD5-1 and 5'Race outer primer. The PCR product was diluted 50-fold and used for the second and third round of amplification with primers PhPOD5-2, PhPOD5-3 and 5'Race inner primer (provided in the kit) respectively. All the primers used in RACE are listed in Table 1.

Bioinformatic analysis and phylogenetic construction: The obtained sequences were analyzed using bioinformatics tools available at the websites http://www.ncbi.nlm.nih.gov and http://www.expasy.org. Similarities analyses were performed using ClustalW in the DNAstar 5.0.221.0 package. N-terminal sequence analysis was carried out by iPSORT and Sigmal IP3.0. Secondary structure analyses were carried out using SWISS-MODEL (http:// swissmodel. expasy.org/). The phylogenetic tree is represented as "Bootstrap N-J tree", obtained with NJ method and edited with TreeExplorer throughout MEGA 3.1.

Semi-quantitative RT-PCR analysis: Semi-quantitative RT-PCR analysis was performed, using the Primescript one-step RT-PCR Kit (code: DRR055A, TaKaRa, Japan) according to the manufacturer's instructions. PCR reactions were performed in a total volume of 20 µl with 1 µl of RNA (50 ng). The PCR conditions for the detection of the *PhPOD* gene were 50°C for 30 min, 94°C for 2 min, followed by 28 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, and a final elongation step of 72°C for 10 min. RT-PCR primers PhPOD3 and PhPOD4 (Table 1) were used to amplify a 392 bp fragment of the *PhPOD* gene. The gene encoding actin was used as a control, with primers Actin-F (5′-CTGAGCGTGAAATTGTAAGGG-3′) and Actin-R (5′-TGCTAAAATAGAACCTCCAATCC-3′). PCR products were analyzed by electrophoresis on a 1.0% (w/v) agarose gel.

Results and Discussion

Using the RT-PCR and RACE method, the full-length cDNA of *PhPOD* (Genbank Accession No.FJ161978) was cloned and confirmed by sequencing. The cDNA has 1251 bp of nucleotides, with an open reading frame (ORF) of 1041 bp, a 43 bp 5'-untranslated sequence and a 167 bp 3'-untranslated region sequence including 22 bp poly (A) sequences. The gene encodes a polypeptide of 347 amino acids.

The deduced PhPOD protein contains 347 aa, with an N-terminal signal peptide of 24 aa (Bendtsen *et al*., 2004). It has a calculated molecular weight (MW) of 37.22 kDa and an isoelectronic point (pI) of 7.55, calculated by the DNAstar software. The putative PhPOD protein has identities of 67%, 66%, 65% and 64% to the peroxidases from *Catharanthus roseus* (CAP72490), *Spinacia oleracea*(CAA71490), *Nicotiana tabacum* (AK52084), *Vigna angularis* (BAA01950), respectively (Fig. 1). Multiple alignment of PhPOD showed that PhPOD contains all of the highly-conserved residues in plant peroxidases, including active residues of Arg70, His74, Asn98 and Pro173 at the distal site and His203 and Asp247 at the proximal site of heme, eight cysteine residues which form four disulfide bridges (43-124, 76-81, 130-325 and 210–237), and two Ca^{2+}-binding sites. PhPOD also contains six putative N-glycosylation sites (Asn-X-Ser/Thr) at the Asn residues 102, 166, 178, 241, and 324 (Fig. 2), predicted by NetNGlyc (http:// www.cbs.dtu.dk/services/NetNGlyc/). These results indicate that PhPOD belongs to the plant

peroxidase family.

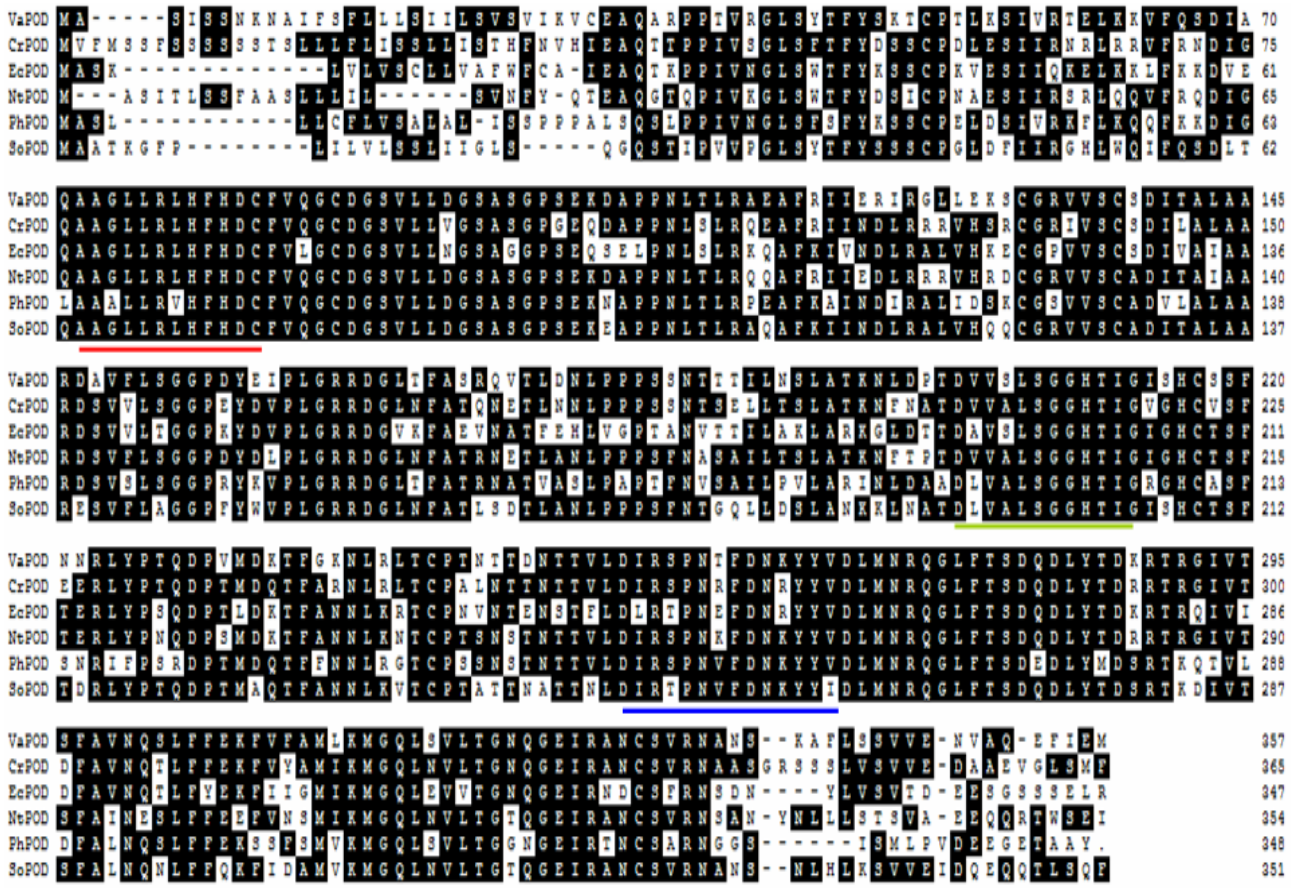

Fig. 1. Alignment of POD sequences
Identical amino acids are highlighted in black, conserved residues for ascorbate, heme and cation Ca are marked with red line and green and blue on the sequences, respectively. Sequences used for comparison and structural analysis of *PhPOD*, the GenBank accession numbers and abbreviations are below: VaPOD (BAA01950) from *V. angularis*; CrPOD (CAP72490) from *C. roseus catharanthus roseus*; EcPOD (AAS97959) from *E. Characias*; NtPOD (AAK52084) from *N. tabacum*; SoPOD (CAA71490) from *S. Oleracea*.

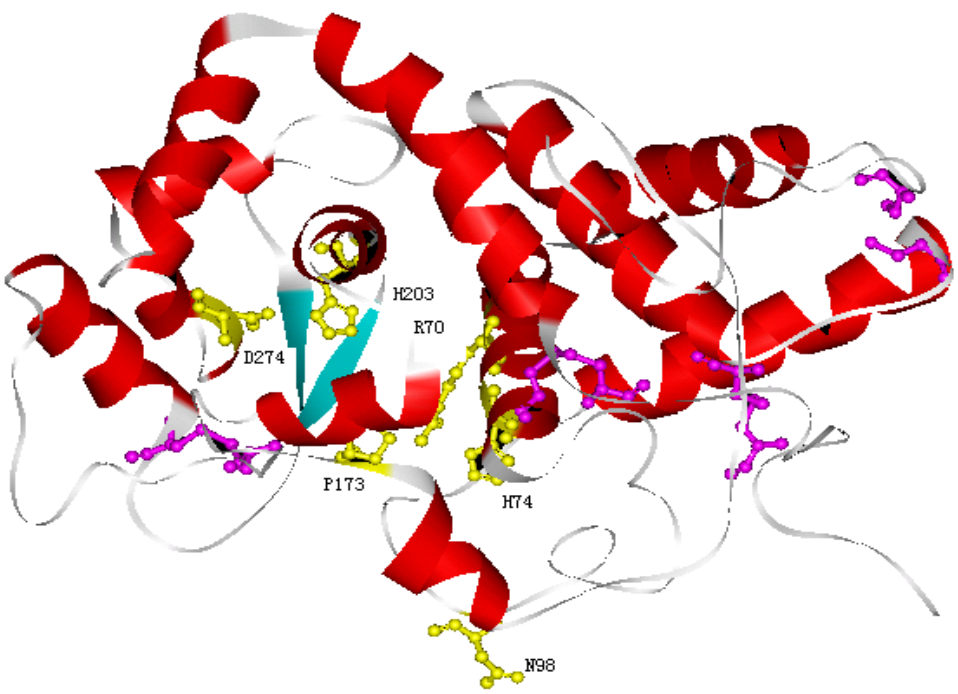

Fig. 2. The tertiary structure of PhPOD predicted with SWISS-MODEL (http://swissmodel.expasy.org) and edited with Weblab Viewerlite 4.0.
The conservative residues of six His (pink) and a Cys (yellow), are signified with line-ball and stick spatial configurations, respectively.

To better characterize the PhPOD protein, a comparative modeling of the 3-D structure of PhPOD was performed using SWISS-MODEL (Schwede *et al*., 2003). Alignments of 3-D structure and structural analyses were carried out using 3-D Molecular Viewer and WEBLAB VIEWERLITE, indicating that all peroxidases aligned had more than ten prominent a-helices at similar positions (Smith & Veitch 1998; Wu *et al*., 2006). As shown in Fig. 2, the predicted structure of PhPOD is predominantly α-helical and some β-sheets. The disulfide bridges which play an important role in stabilizing the structure of peroxidase were also found in PhPOD.

A phylogenetic tree of peroxidases (Fig. 3) shows that PhPOD, CrPOD, CoPOD, CsPOD, NtPOD, VaPOD, PgPOD are classified into one large branch, revealing their close evolutionary relationship which points to a similar physiological function.

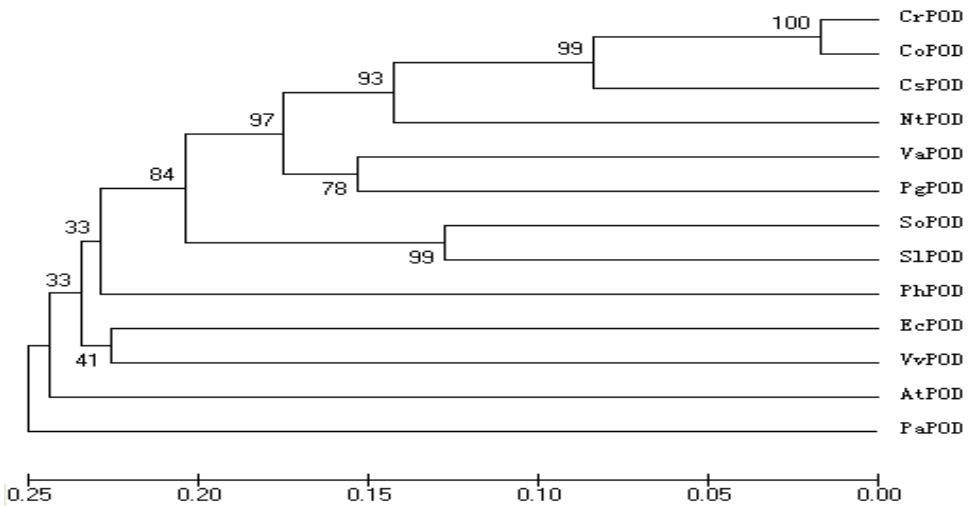

Fig. 3. Phylogenetic tree analysis of PODs from different plant species.
The analysis was based on the deduced amino sequences of CrPOD (CAP72490) from *C. roseus catharanthus roseus*; CoPOD (CAP72489) from *C. roseus catharanthus roseus*; CsPOD (CAJ84723) from *C. roseus*; NtPOD (AAK52084) from *N. tabacum*; VaPOD (BAA01950) from *V. angularis* ; PgPOD (BAE20169) from *P. ginseng*; SoPOD (CAA71490) from *S. Oleracea*; SlPOD (CAA71488) from *S. Oleracea*; EcPOD (AAS97959) from *E. Characias*;VvPOD (CAO43597) from*V. Vinifera*; AtPOD (NP177313) from *A. thaliana*; PaPOD (CAD92856) from *P. abies*.

Many studies revealed that peroxidase can be induced by a variety of stresses or elicitors such as cold, MeJA, H_2O_2 and so on (Almagro *et al*., 2009; Sosio *et al*., 2009). Our previous study showed that the activity of POD increased and novel POD isoforms were found before *Phalaenopsis* explant browning (Xu *et al*., 2006). Analysis by semi-quantitative RT-PCR showed that the expression of *PhPOD* was increased gradually in the first 6 h and remained at a high level during 6 d cultured after being cut from the plant. The mRNA level of *PhPOD* in explant declined in 12 d compared to that of 6 d (Fig. 4). We have found the brown was occurrence after 3 d culture at the edge of explant and turn serious after 6 d culture. The explants turn brown and then cause cell death. The activities of POD were high before explant browning occurrence and decline when the explant browning intensified (Xu & Li, 2006). These results suggest that there is a consistent relationship between the POD activity and *POD* gene expression.

The classic plant PODs belong to a large multigene family and take part in a wide range of physiological processes, including lignification, suberization, cross-linking of cell-wall proteins, auxin metabolism, defence against pathogen attack and oxidative stress (Hiraga *et al*., 2001). Both POD and PPO catalyse the conversion of plant diphenols to highly reactive quinones. These quinones bind to electron-rich moieties of amino acids and proteins yielding a complex mixture of brown products (Pource *et al*., 2007). Lagrimini (1991) demonstrated that transgenic plants overexpressing acidic PODs showed more rapid wound induced browning of tissues than control plants. Our results illustrate that the increased POD activity may be due to the high level of *POD* expression which causes explant browning and tissue necrosis.

In summary, a full-length cDNA of a peroxidase gene from *Phalaenopsis* was cloned and characterized. Multiple alignments show that the deduced PhPOD is highly homologous to other peroxidase proteins, and that PhPOD contains all conserved residues in plant peroxidase protein family. The *PhPOD* gene expression was increased after 6 h culture. This is the first report of the changes of POD gene expression during explant browning. This study provides a new view for the explant browning and may bring a more economical and effective way to inhibit explant browning.

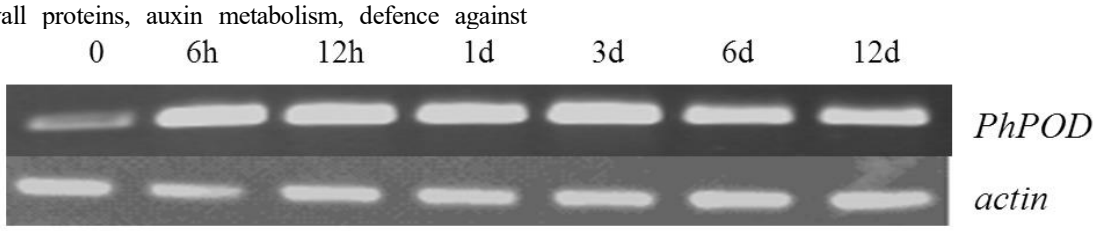

Fig. 4. RT-PCR analysis of the expression of *PhPOD* mRNA during *Phalaenopsis* explant browning cultured for 12 days.

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