Mycorrhiza (2010) 20:201–207
DOI 10.1007/s00572-009-0270-1

SHORT NOTE

Fast track in vitro mycorrhization of potato plantlets allow studies on gene expression dynamics

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Received: 5 June 2009 / Accepted: 24 July 2009 / Published online: 2 September 2009

Abstract Root colonization by arbuscular mycorrhizal (AM) fungi is a dynamic process involving major changes in plant gene expression. Here, the expression of a phosphate transporter gene (*PT3*) and several defense genes, already known to be involved in the various stages of AM establishment, were monitored in the mycelium donor plant (MDP) in vitro culture system associating potato plantlets with an AM fungus. This system allows fast and homogenous mycorrhization of seedlings at their early stage of development by growing the plantlets in active mycelial networks, but has never been validated for gene expression analysis. Here, QRT-PCR analyses were conducted in parallel to pre- (1 day), early (2 and 3 days), and late (6, 9, and 15 days) stages of root colonization. We observed the induction of a plant gene marker of AM root colonization (*PT3*) at the late stage and the induction of *MAPK* and *PAL* genes at the early and late stages of root colonization. We also demonstrated the induction of *PR1* and *PR2* genes at pre- and late stages and of *GST1* and *Lox* genes at a late stage of root colonization. These results validated the MDP in vitro culture system as an optimal tool to study gene expression analysis during the AM fungi establishment. This system further opened the door to investigate gene networks associated with the plants–AM fungi symbiosis.

Keywords Gene expression · *Glomus intraradices* · In vitro system · Mycelium network · *Solanum tuberosum*

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Introduction

Arbuscular mycorrhizal (AM) symbiosis represents a unique association between the mycelium of a soil-borne fungus and more than 80% of higher plants. The fungus, an obligate biotroph belonging to the phylum Glomeromycota (Schuβler et al. 2001), receives carbohydrates required to complete its life cycle from the host plant; in exchange, it provides the plant with nutrients, such as phosphate. The establishment of this successful mutualistic association develops from a complex and dynamic process involving major changes in fungal and plant gene expression (Franken et al. 2007; Reinhardt 2007). During this process, phosphate transporter genes (Liu et al. 1998) as well as transient defense-response genes are activated in the host plant (Liu et al. 2003).

In the last few years, in vitro cultivation systems associating excised root organs with AM fungi have been used to investigate gene expression (González-Guerrero et al. 2005; Elfstrand et al. 2005; Waschke et al. 2006). These systems present several advantages, among which are the absence of undesirable microorganisms and the possibility to nondestructively monitor the development of the fungal colony. However, some limitations are also associated to these systems, materialized by the absence of photosynthetic tissues: a normal hormonal balance and physiological source-sink relationships (Fortin et al. 2002). In addition,

the roots used in these experiments are, most often, genetically transformed organs.

Recently, Voets et al. (2005) and Dupré de Boulois et al. (2006) developed two in vitro autotrophic culture systems associating, respectively, potato and *Medicago truncatula* to root organ culture (ROC)-produced AM fungal spores. Mass production of spores (i.e., approximately 12,000 in 22 weeks) was obtained with potato (Voets et al. 2005), and the transport of C from the shoot of *M. truncatula* to the fungus (Voets et al. 2008) and of P and C from a root-free labeled compartment to the shoot via the extraradical mycelium (Dupré de Boulois et al. 2006) was demonstrated. However, with both systems, high-level colonization took several weeks, and nearly full-grown mycorrhizal plants were produced before sampling. This may hinder experiments on gene expression analysis in plantlets during the early stages of root colonization. It is obvious that any system allowing the fast and homogenous mycorrhization of seedlings within a few days is highly desirable.

In nature, the AM fungal mycelium growing from colonized roots represents an important source of inoculum for the colonization of neighboring plants due to the several hyphal apices ramifying from the colony (Friese and Allen 1991). Recently, Voets et al. (2009) have developed a new in vitro mycorrhization system adapted to seedlings by using the symbiotic phase of the fungus as inoculum for fast and homogenous AM colonization. This mycelium donor plant (MDP) in vitro culture system was successfully applied to *M. truncatula* plants opening large perspectives to study various aspects of the AM fungi and AM symbiosis.

In this study, we validated the MDP in vitro culture system of Voets et al. (2009) for gene expression analysis. The expression of a phosphate transporter gene (*PT3*) and several defense genes, already well known to be involved in the various stages of AM establishment, were analyzed at six time points corresponding to the establishment of *Glomus intraradices* in roots of potato plantlets by combining the quantitative real-time-polymerase chain reaction (QRT-PCR) analyses with the assessment of intraradical root colonization.

Materials and methods

Biological material

Propagation and maintenance of stock of potato plantlets

Potato plantlets propagated in vitro (*Solanum tuberosum* L., var. Bintje) were supplied by the Station de Haute Belgique in Libramont, Belgium. Plantlets were micropropagated every 5 weeks as described in Voets et al. (2005).

Culture, propagation, and maintenance of G. intraradices

A root organ culture of *G. intraradices* Schenck and Smith MUCL 41833 was supplied by GINCO (http://www.mbla.ucl.ac.be/ginco-bel). The spores were extracted by solubilization of the gellan gel (Doner and Becard 1991) and approximately 100 were placed in the near vicinity of actively growing transformed carrot (*Daucus carota* L.) roots (approximately 70 mm in length) on Petri plates (90 mm in diameter) containing the modified Strullu–Romand (MSR) medium (Declerck et al. 1998 modified from Strullu and Romand 1986), solidified with 3 g l^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA; Cranenbrouck et al. 2005). The Petri plates were incubated for 3 months in the dark in an inverted position at 27°C, and several thousand spores were produced during this period.

M. truncatula seed disinfection

Seeds of *M. truncatula* Gaertn. cv. Jemalong A 17 (SARDI, Australia) were surface-sterilized by immersion in sodium hypochlorite (8% active chloride) for 12 min, rinsed three times in deionized sterile water and germinated in groups of 25 on Petri plates (90 mm in diameter) filled with 35 ml MSR medium without sucrose or vitamins, and solidified with 3 g l^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA). Petri plates were incubated at 27°C in the dark.

Experimental design

Micropropagated potato plantlets were plated on actively growing extraradical mycelium networks of AM fungi developing in the hyphal compartment (HC) of bicompartmental Petri plates (for details, see Voets et al. 2009). The time course of gene expression was analyzed during symbiotic establishment in potato roots.

Eight weeks after association, a dense extraradical mycelium bearing numerous spores developed in the HC. The length of mycelium was 6,261 cm±2367 and the number of spores, 19,385±9,710 (estimated following the method of Voets et al. 2005).

Two new holes (±2-mm diameter), separated by a 4.5-cm distance from each other, were then made in the base and the lid of the Petri plates at the side of the HC. One ten-day-old potato plantlet was inserted in each hole following the same methodology described by Voets et al. (2009) with their roots in direct contact with the extraradical mycelium. The Petri plates were then sealed carefully and incubated in a growth chamber under controlled conditions, that is, 22/18°C (day/night), 70% relative humidity, photoperiod of 16 h d^{-1}, and an average photosynthetic photon flux of 225 μmol m-2 s^{-1}. Identically, two micropropagated potato plantlets were

placed in the HC of the control treatment (i.e., noninoculated 4-day-old *M. truncatula* seedlings) and grown under the same conditions as described previously.

Twenty-four cultural systems were randomly divided into 6 groups of four replicates. Roots were harvested 1, 2, 3, 6, 9, and 15 days after contact (dac) with the extraradical mycelium network of *G. intraradices*. One control plant was also harvested each time. For each cultural system, the two potato plantlets from the HC were pooled to obtain sufficient material for analysis. Half of the material was then used to estimate intraradical root colonization while the other half was used for gene expression analysis.

Intraradical root colonization

The intraradical root colonization was estimated under a dissecting microscope (Olympus SZ40, Olympus Optical GmbH, Hamburg, Germany) at ×10 to ×40 magnification, according to McGonigle et al. (1990). Roots were cleared in 10% KOH at 50°C for 90 min, rinsed with distilled water, and stained with trypan blue 1% (Phillips and Hayman 1970) at 50°C for 60 min. Percentages of root colonization were subjected to one-way analysis of variance (ANOVA). Tukey's Honest Significant Difference (HSD) was conducted to identify significant differences ($P \leq 0.05$) between the groups. Data analysis was performed with the SAS enterprise guide 4.1 (SAS Institute Inc., Cary, USA).

RNA extraction

Total RNA was extracted from 50 to 100 mg frozen material with Trizol® reagent (Invitrogen, Carlsbad, USA) with an additional chloroform purification step and then purified using the Purelink™ Micro-to-Midi total RNA purification system (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The total RNA was subsequently treated with the TURBO DNA-free™ kit (Ambion, Austin, USA) according to the manufacturer's instructions. Concentration and purity of total RNA were determined in a NanoDrop®-ND 1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA) using a 2-µl aliquot of the total RNA solutions. RNA purity was estimated from the A260/A280 absorbance ratio.

Reverse transcription

Following total RNA extraction, reverse transcription (RT) of 500 ng of RNA was performed with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Montreal, Canada) in a volume of 20 µl with Oligo$(dT)_{18}$ primer at 55°C for 20 min according to the manufacturer's instructions. For each RNA sample, a reaction without RT was performed as a control for contamination by genomic DNA.

Primer design

Four reference genes: glyceraldehyde phosphate dehydrogenase (*GAPDH*), ubiquitin conjugating enzyme-like (*Ubc*), elongation factor 1-alpha (*EF1-α*), and beta-tubulin (*β-tub*) (Nicot et al. 2005) and seven genes: gluthatione-s-transferase 1 (*GST1*), lipoxygenase (*Lox*), MAP kinase (*MAPK*), Pathogenesis Related 1 (*PR1*), Pathogenesis Related 2 (*PR2*), phenylalanine ammonia lyase (*PAL*), and phosphate transporter 3 (*PT3*) were selected. Potato nucleotide sequences were obtained from the GenBank database. Eleven primer pairs were designed from these sequences (90–110-bp length, optimal temperature of annealing at 60°C, GC% between 40% and 60%) with the LightCycler Probe Design Software 2.0 (Roche, Montreal, Canada). The forward and reverse primer sets and melting temperatures (in brackets) were as follows: 5′-CCAAGTAA CCTCTTGCTAAATGC-3′ and 5′-CTGTCATATTCTC GTTCTCTAGG-3 for *MAPK* (79°C); 5′-GCTTTGC TTACTTATTATTGGCG-3′ and 5′-GGAAGCAGCCT TAGTAGCATT-3′ for *PT3* (82°C); for the nine other primer sets, see Gallou et al. (2009).

Quantitative real-time PCR

QRT-PCR analysis was performed using the LightCycler 2.0 (Roche, Montreal, Canada). A set of standard solutions prepared from RT products was included in each run. Reactions were prepared in capillaries using the following concentrations: 7 µl of PCR water, 4 µl of 5× LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Mix (Roche, Montreal, Canada), 2 µl of each forward and reverse primer (0.5 µM), and 5 µl of 1:10 diluted cDNA or standard solution as template (for LightCycler experimental run, see Gallou et al. 2009). In order to check PCR efficiency, standard curves (log of cDNA dilution versus C_p) using serial 10-fold dilution of cDNA were created for each pair of selected primers. To obtain good comparison and normalization, PCR efficiency should range between 80% and 115%. In this study, all the PCRs displayed efficiencies between 91% and 104%. For the mathematical model, it was necessary to determine the crossing point (Cp) for each transcript, defined as the point at which the fluorescence rises appreciably above the background fluorescence. The Fit point method was performed in the LightCycler software 4.1 at which Cp was measured at a constant fluorescence level. The combination of several reference genes smooths out normalization error due to the small variation in the expression of a single reference gene (Vandesompele et al. 2002). We determined the best combination of reference genes for normalization of gene expression by using the geNorm software (http://medgen.ugent.be/~jvdesomp/geNorm). The most stable genes

expressed in the potato roots during symbiotic establishment with *G. intraradices* were *β-tub* and *EF1-α*. The pairwise variation demonstrated that three reference genes (i.e., *β-tub*, *EF1-α*, and *GAPDH*) were sufficient to normalize gene expression in potato plantlets grown in the extraradical mycelium network of *G. intraradices* (data not shown). Normalization was achieved using the geometric means of the three reference genes, i.e., *β-tub*, *EF1-α*, and *GAPDH*. The data were analyzed statistically with software REST 2008 (relative expression software tool; Pfaffl et al. 2002; http://rerst.gene-quantification.info/). Significance values were set at P value <0.05. P(H1): the probability of alternate hypothesis that differentiates between sample and control groups is due only to chance.

Results

Time course of root colonization of potato plantlets grown in the extraradical mycelium network of *G. intraradices*

The intraradical root colonization of potato plantlets was assessed 1, 2, 3, 6, 9, and 15 dac of the root system with the AM fungal extraradical mycelium network (Table 1). No AM root colonization was observed 1 dac with the extraradical mycelium network. The first traces of AM fungal root colonization were detected 2 dac (1.9±3%) and increased slightly (9.9±7%) 3 dac. However, no significant differences were observed between the values recorded at 1, 2, and 3 dac. Neither arbuscules nor vesicles were observed at 1, 2, and 3 dac. Six dac, the AM root colonization increased markedly (24±24%), and the first arbuscules and vesicles were observed. The percentages of arbuscules and vesicles observed 6 dac were 3.9 and 0.3, respectively. The AM root colonization estimated 9 dac was 48.2±14% and remained almost identical 15 dac (49.9±8%). The values of AM root colonization were significantly higher, 9 and 15 dac as compared with 1, 2, and 3 dac. The 6 dac values recorded were intermediate between those obtained 1, 2, 3 dac and 9 and 15 dac. At 9 dac, the percentages of arbuscules and vesicles were 13.6 and 3.5, respectively. At 15 dac, the percentages of arbuscules and vesicles were 8.2 and 5.7, respectively.

Expression of marker gene of AM root colonization of potato plantlets grown in the extraradical mycelium network of *G. intraradices*

The relative expression ratio of *PT3* genes in potato roots was assessed 1, 2, 3, 6, 9, and 15 dac with the extraradical mycelium network (Table 2). We observed an induction of *PT3* gene 9 and 15 dac with a maximum of 15 dac [65.30 dac (19.90–191.08 dac)].

Expression of defense response genes in potato plantlets grown in the extraradical mycelium network of *G. intraradices*

The relative expression ratio of the defense response genes *GST1*, *Lox*, *MAPK*, *PAL*, *PR1*, and *PR2* in potato roots was assessed 1, 2, 3, 6, 9, and 15 dac with the extraradical mycelium network (Table 2). For the *GST1* gene, the induction of 6, 9, and 15 dac was detected with a maximum of 15 dac [5.68 dac (4.20–8.76 dac)]. For the *Lox* gene, the induction of 9 and 15 dac was detected with a maximum of 15 dac [6.12 (2.73–10.13)]. The *MAPK* and *PAL* genes were induced 2 dac, and an induction was noted 6, 9, and 15 dac with a maximum of 15 dac [8.12 dac (3.92–16.60 dac)] for *MAPK* and [28.55 (11.40 – 60.85)] for *PAL*. For the two Pathogen Related genes (i.e., *PR1* and *PR2*), an induction of 1 dac was detected, and respectively 9 and 15 dac with a maximum of 15 dac [105.31 dac (60.33–229.85 dac)] for *PR1*, and 6, 9, and 15 dac with a maximum of 15 dac [159.94 (85.56 – 330.10)] for *PR2*.

Discussion

Root organ cultures have been considered as suitable systems to investigate various aspects of the AM fungi

Table 1 Intraradical colonization of potato roots plated on actively growing extraradical mycelium networks of *G. intraradices*

		Intraradical root colonization during the time course[a]					
		1	2	3	6	9	15
Colonization (%)	Total	0 (±0) a	1.9 (±3) a	9.9 (±7) a	24 (±24) ab	48.2 (±14) b	49.9 (±8) b
	Arbuscules	0	0	0	3.9	13.6	8.2
	Vesicles	0	0	0	0.3	3.5	5.7

Values of colonization followed by different letters differ significantly at $P \leq 0.05$ (one-way ANOVA and Tukey's HSD). The standard error is shown in brackets (biological replicates = 4)

[a] Days after contact of potato plantlets with the network of *G. intraradices*

Table 2 Relative expression ratio of seven genes (gluthatione-s-transferase 1 (*GST1*), lipoxygenase (*Lox*), MAP kinase (*MAPK*), Pathogenesis Related 1 (*PR1*), Pathogenesis Related 2 (*PR2*), phenylalanine ammonia lyase (*PAL*), and phosphate transporter 3 (*PT3*)) in potato roots plated on actively growing extraradical mycelium networks of *G. intraradices* normalized by the geometric mean of the three reference genes (i.e., *β-tub*, *EF1-α*, and *GAPDH*)

Gene name	Relative expression ratio during the time course[a]					
	1	2	3	6	9	15
PT3	1.86 [0.44–9.71]	0.24 [0.08–0.94]	1.81 [1.05–2.56]	0.28 [0.05–4.00]	*5.51 [2.58–16.02]*	*65.30 [19.90–191.08]*
GST1	1.40 [1.01–2.14]	1.41 [0.93–2.70]	0.30 [0.19–0.46]	*2.72 [2.39–3.10]*	*4.48 [2.93–7.47]*	*5.68 [4.20–8.76]*
Lox	1.00 [0.40–3.71]	0.63 [0.10–2.08]	0.62 [0.47–0.84]	1.80 [1.02–4.51]	*3.38 [1.26–7.60]*	*6.12 [2.73–10.13]*
MAPK	0.64 [0.17–2.09]	*5.74 [1.75–16.78]*	0.42 [0.32–0.56]	*2.64 [1.70–4.01]*	*2.01 [1.30–3.95]*	*8.12 [3.92–16.60]*
PAL	1.95 [0.83–5.11]	*7.82 [3.22–21.04]*	3.88 [1.52–14.37]	*12.10 [5.32–30.22]*	*3.06 [1.26–11.25]*	*28.55 [11.40–60.85]*
PR1	*2.50 [1.60–4.79]*	0.15 [0.05–0.69]	1.05 [0.70–1.63]	1.21 [0.55–2.31]	*7.35 [1.40–57.06]*	*105.31 [60.33–229.85]*
PR2	*3.94 [1.57–10.30]*	1.63 [0.67–3.47]	1.80 [0.98–3.27]	*13.39 [6.07–29.52]*	*10.51 [1.35–194.15]*	*159.94 [85.56–330.10]*

Data were analyzed statistically by the software REST 2008 (relative expression software tool; Pfaffl et al. 2002; http://rerst.gene-quantification.info/). Upregulated genes (*in italics*) with significance values were set at P value <0.05 (P(H1): the probability of alternate hypothesis that differentiates between sample and control groups is due only to chance). The standard error range is shown in brackets (biological replicates = 4)

[a] Days after contact of potato plantlets with the extraradical mycelium network of *G. intraradices*

and AM symbiosis (Fortin et al. 2002). Recently, autotrophic nontransformed plants have been successfully cultured in vitro in association with AM fungi (Voets et al. 2005; Dupré de Boulois et al. 2006), and a fast-track in vitro mycorrhization system (i.e., the MDP in vitro culture system) was developed (Voets et al. 2009). This system opens large perspectives for molecular studies but should be validated for gene expression analysis. Here, the expression of a phosphate transporter gene and six defense genes were monitored by QRT-PCR and paralleled with the multistep process of potato AM fungal root colonization. Gene expression change could be observed in prestage (before root colonization, 1 dac), early stage (hyphal root colonization before arbuscule and vesicle formation, 2 and 3 dac), and the late stage of root colonization (intense root colonization with arbuscules and vesicles formed, 6, 9, and 15 dac).

During the precolonization stage of the potato–*G. intraradices* interaction (1 dac), we observed the induction of *PR1* and *PR2* genes while no induction was noted during the following 2 and 3 dac (early root colonization stage). This change in gene expression between pre- and early stages of AM fungi root colonization was reported earlier by Kapulnik et al. (1996), Ruíz-Lozano et al. (1999), and Liu et al. (2003) and associated with a transient level of defense-gene expression.

During the early stage of the potato–*G. intraradices* interaction (2 and 3 dac), we observed the induction of the *MAPK* gene (at 2 dac). This observation is consistent with the role of MAP kinases in the signaling of plant abiotic stress and pathogen defense (Nakagami et al. 2005) and corroborates the findings of Deguchi et al. (2007). The early stage of root colonization was also characterized by the induction of *PAL* gene as reported by Deguchi et al. (2007).

During the late stage of the potato–*G. intraradices* interaction (6, 9, and 15 dac), we observed an induction of *PR1* (9 dac) and *PR2* (6 dac) with a buildup in the level of induction, paralleled thereafter with increased root colonization. *PR1* gene is a PR protein with antifungal properties and an unknown microbial target (Antoniw et al. 1980). This gene is known to respond to fungal infection in potato (Gallou et al. 2009). *PR2* gene is a β-1,3-glucanase induced, among others, in *M. truncatula* roots colonized by *G. intraradices* and *Glomus mosseae* during the late stage of root colonization (Hohnjec et al. 2005).

We noted the induction of a *Lox* gene (i.e., the first enzyme in the biosynthesis pathway of JA gene) 9 dac. This is in agreement with earlier results showing that, in roots of mycorrhizal plants, the levels of JA were higher as compared with nonmycorrhizal controls (Meixner et al. 2005; Stumpe et al. 2005; Hause et al. 2007). We also observed a significant induction of *PAL* gene 6, 9, and 15 dac with increased percentages of arbuscules and vesicles in roots. Harrison and Dixon (1994) have demonstrated that transcripts encoding enzymes of the isoflavone biosynthetic pathway, such as *PAL* and chalcone synthase, are induced specifically in cells containing arbuscules.

Glutathione-s-transferase transcripts have been found to accumulate in roots containing arbuscules (Wulf et al. 2003; Brechenmacher et al. 2004). In our study, we observed the induction of the *GST1* gene in the potato roots 6, 9, and 15 dac in parallel with the formation of the first arbuscules and vesicles. We also observed the induction of an *MAPK* gene in the late stages of root colonization, as reported by Weidmann et al. (2004) and

Grunwald et al. (2004) in *M. truncatula* roots colonized by *G. mosseae.*

During the late stage of the potato–*G. intraradices* interaction (6, 9, and 15 dac), we observed the induction of the phosphate transporter gene *PT3* (9 dac) with a buildup in the level of induction, 15 dac, paralleled with an increase in root colonization. Rausch et al. (2001) have identified the phosphate transporter gene *StPT3* in potato and localized *PT3* induction specifically in arbuscule-containing cells.

During this study on gene expression analysis at the different stages of AM establishment with the MDP in vitro culture system, we observed the induction of *PT3* gene at the late stage of potato–*G. intraradices* interaction. Two PR genes (*PR1* and *PR2*) were induced prior to root colonization with a transient expression at 2, 3, and 6 dac (for *PR1*) followed by a continuous buildup in the level of induction. We finally demonstrated the induction of *GST1*, *Lox*, *MAPK*, and *PAL* genes at different stages of potato–*G. intraradices* interaction. The result obtained for the *PT3* gene (a plant gene marker of AM root colonization) demonstrated that the potato–*G. intraradices* association was successfully established and the mutualistic exchange between the two partners was effective. The induction of defense genes, well known to be involved at different stages of the AM symbiosis, demonstrated that the potato–*G. intraradices* establishment in the MDP in vitro culture system was suitable to study major changes in plant gene expression and corroborated previous results of in vivo studies. Our study opened new avenues to investigate the molecular events or gene networks associated with the plants–AM fungi symbiosis by making it possible to synchronize the development of AM fungi in the roots of plants grown in an established nonperturbed mycelium network under rigorous in vitro culture conditions.

Acknowledgements This research was sponsored by the Direction Générale de l'Agriculture de la Région wallonne under contract number D31-1149, entitled *Valorisation de la microflore bénéfique des sols pour le contrôle de la flore pathogène des productions de pomme de terre comme alternative à l'utilisation des pesticides*. S. C. gratefully acknowledges the financial contribution of the Belgian Federal Science Policy Office (contract BCCM C3/10/003).

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