Cloning and characterization of CaGID1s and CaGAI in Capsicum annuum L.

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Abstract

Fruit set and development are affected by many phytohormones, including gibberellin. Little is known regarding molecular mechanism underlying gibberellin mediated fruit set and development especially in Capsicum. Three gibberellin receptors, CaGID1b.1, CaGID1b.2 and CaGID1c, and a DELLA protein, CaGAI, have been identified in Capsicum annuum L. During the fruit development, the expression level of CaGID1c was low, and the expression fold change is mild. However, CaGID1b.1 and CaGID1b.2 were relatively higher and more acute, which indicates that CaGID1b.1 and CaGID1b.2 may play an important role in fruit pericarp, placenta and seed. Ectopic expressions of CaGID1b.1, CaGID1b.2 and CaGID1c in Arabidopsis double mutant gid1a gid1c increased plant height, among which CaGID1b.2 had the most significant effect; CaGAI reduced plant height in double mutant rga-24/gai-t6, having a similar function to AtGID1 and AtGAI in stem elongation. Yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays indicated that CaGID1b.1 and CaGID1b.2 interact with CaGAI in a GA-dependent manner, while CaGID1c interacts with CaGAI in a GA-independent manner. Our study reveals the key elements during gibberellin signaling in Capsicum and supports the critical importance of gibberellin for Capsicum fruit set and development.

Keywords: Capsicum annuum L., fruit, GID1, DELLA

1. Introduction

Successful pollination and fertilization is necessary for fruit set and development in most flowering plants (Gillaspy et al. 1993), and failure of fertilization leads to seedless fruit or abscission in most peppers (Capsicum annuum L.) (Tiwari et al. 2013).

Plant hormones are the main regulators of fertile fruits (Gillaspy et al. 1993). Therefore understanding the regulation of plant hormones on fruit set and development is of considerable agronomic value (Kang et al. 2013). Auxin was considered one of the most important hormones regulating fruit set and development (Nitsch 1950; Vriezen et al. 2008; Tiwari et al. 2013; Vargas et al. 2013). Later the function of gibberellin was also identified in the following investigations (Yamaguchi and Takahashi 1976; Bukovac et al. 1979; Mesejo et al. 2013), which was thought to be
downstream of auxin that affected fruit set in pea, tomato and pepper (Ngo et al. 2002; Ozga et al. 2003; Serrani et al. 2008; Tiwari et al. 2012), and it alone or in combination with auxin stimulated fruit set and development in the absence of pollination (Thompson 1969).

Gibberellin affects plant growth and development in a variety of aspects, including seed germination, hypocotyls elongation, root growth, leaf expansion, stem elongation, trichome formation, floral induction, flower development, fruit set and fruit development (Hooley 1994; Ross et al. 1997; Fleet and Sun 2005; Tiwari et al. 2012). GA enhances organ size by promoting cell elongation and cell division (Achard et al. 2009; Ubeda-Tomás et al. 2009). GibBERELLIN INSENSITIVE DWARF1 (GID1) is gibberellin receptor, and three GID1 genes were identified in Arabidopsis, tomato, apple and grape, respectively, while only one was identified in rice, barley and maize, respectively (El-Sharkawy et al. 2014). GID1 binds to GA and then triggers a series of responses promoting plant growth. DELLA is a repressor in the GA signaling pathway, which belongs to a GRAS subfamily and there are DELLA and VHYNP motifs at the N-terminal region of all DELLA proteins (Peng et al. 1998; Bolle 2004; Hussain et al. 2005). Five members of DELLA (REPRESSOR OF ga1-3 (RGA), GIBBERELLIN INSSENSITIVE (GAI), RGA-LIKE 1 (RGL1), RGA-LIKE 2 (RGL2) and RGA-LIKE 3 (RGL3)) (Olszewski et al. 2002; Fleet and Sun 2005) were identified in Arabidopsis, while only one DELLA was identified in rice, barley, maize and tomato, respectively (El-Sharkawy et al. 2014). GID1 binds to GA and then triggers a series of responses promoting plant growth.

A model of GA signaling has been constructed as below: when GA is present, GID1, the receptor of GA binds to GA and leads to the formation of GA-GID1 complex, stimulating interaction between GID1 and DELLA, and causes conformation change of DELLA, which enables recognition of DELLA by SCF[SBY1/GID2] and then leads to degradation of DELLA protein by ubiquitin degradation. The degradation of DELLA protein switches on gene expression for plant growth and development (Richards et al. 2001; Gomi and Matsuoka 2003; Sun and Gubler 2004; Yasumura et al. 2007; Anizumi et al. 2008; Hirano et al. 2008; Gao et al. 2011; Kang et al. 2013).

In Capsicum, a variety of factors influencing fruit set and development have been studied, including plant hormones (Huberman et al. 1997; Heuvelink and Körner 2001), assimilate availability (Marcelis et al. 2004), assimilate utilization and dominance of competing fruits (Aloni et al. 1996), reduced metabolic activity (Aloni et al. 1995), but some factors including plant hormones have not been well investigated at the molecular level. In this study, we aim to investigate the characters and function of gibberellin signaling element genes GID1 and DELLA in Capsicum fruit, and make exploration of their interaction.

2. Results

2.1. CaGID1s and CaGAI sequences analysis

Three putative GID1 orthologs—CaGID1b.1, CaGID1b.2 and CaGID1c, and a DELLA gene CaGAI (62% identical to AtGAI and 61% identical to SgGAI) were identified in Capsicum. Their sequences were submitted to GenBank. The accession numbers are: CaGID1b.1, KT369115; CaGID1b.2, KT369116; CaGID1c, KT369117; CaGAI, KT369118. Comparing the cDNA sequences of CaGID1b.1, CaGID1b.2, CaGID1c and CaGAI obtained from Capsicum annuum L. cv. 0819 with those corresponding ones from Capsicum annuum L. cv. Zunla-1, only one mismatch exists at the 942th nucleotide of CaGID1c. In 0819, it is a guanylic acid, but in Zunla-1, it is an adenylic acid, while no amino sequence change occurs (both of them encode lysine).

As shown in Fig. 1, the dendrogram divided the dicotyledon and monocotyledon into two main clades, and divided the dicot proteins into two main clades with high bootstrap values. These three CaGID1s were classified according to phylogenetic tree constructed by ClustalW in MEGA5.05. CaGID1b.1 and CaGID1b.2 were located in GID1b group, and CaGID1a was located in GID1ac group. The DELLA protein was defined as CaGAI due to its homology to SIGAI and AtGAI.

The full length CaGAI cDNA consists of 1728 bp and encodes 76 amino acids without intron in the gene. The conserved two domains DELLA and VHYNP are located in the N-terminal region of CaGAI, which are supposed to be important for GA signaling response, and a GRAS subfamily is located in the C-terminal region, and all of these are supposed to be necessary for DELLA protein.

2.2. Expression patterns of CaGID1s and CaGAI

Upon comparison of gene relative expression of CaGID1s and CaGAI in pollinated and un-pollinated fruits, higher expression levels were found in un-pollinated at 5 days after pollination (DAP) with an exception of CaGAI in pericarp (Fig. 2). This may be related to the small rise of active GAs content in placenta and seed of un-pollinated fruit, and CaGID1b.1 expressed most highly in seed of un-pollinated fruit, and CaGID1b.2 expressed most highly in pericarp and placenta of un-pollinated fruit, therefore CaGID1b.2 may play the most important role among CaGID1s in the growth of pericarp and placenta.
Fig. 1 Phylogenetic tree of GID1s. The phylogenetic analysis was constructed based on the neighbor joining method. Homologs of plant GID1 from 5 dicotyledon and 3 monocotyledon species, Capsicum annuum CaGID1b.1, CaGID1b.2, CaGID1c; Arabidopsis thaliana AtGID1a (NP_187163), AtGID1b (NP_191860), AtGID1c (NP_198084); Vitis vinifera VvGID1a (AFG17072), VvGID1b (XP_002271700), VvGID1c (XP_002265764); Malus domestica MdGID1b.1 (AFD32891), MdGID1b.2 (AFD32882), MdGID1c (AEC04638); Solanum lycopersicum SgGID1b.1 (XP_00424525), SgGID1b.2 (NP_00134767), SgGID1c (XP_004230154); Oryza sativa OsGID1 (NP_001055520); Zea mays ZmGID1 (CAP64327); Hordeum vulgare HvGID1 (CAO98733) were used for analysis. Bootstrap confidence values are labeled above the branches. The three members of CaGID1s are indicated with ▲.

Fig. 2 Relative expression of CaGID1s and CaGAI in different Capsicum tissues 5 days after anthesis with or without pollination. Pe, pericarp; Pl, placenta; S, seed; N, non-pollination. The same as below.
during fruit set; while CaGID1b.1 was the most important among CaGIDs in seed during fruit set.

The expression patterns of CaGID1b.1, CaGID1b.2, CaGID1c and CaGAI are similar during fruit development in three tissues—pericarp, placenta and seed, respectively (Fig. 3). They express high at 0 DAP, and fall down at 5 and 10 DAP when the fruits showing the fastest relative growing rate (data not shown), and then increase at 50 DAP when the fruits almost stop growing (data not shown), with the exception of CaGAI in seed, which shows a slight rise up from 0 to 50 DAP.

In pericarp, CaGID1b.2 showed the highest expression level at 0 DAP, while CaGID1b.1 decreased 8.06 times, which was the highest fold change among CaGIDs from 0 to 5 DAP; in placenta, CaGID1b.2 both had the highest expression level and fold change of 10.00 from 0 to 5 DAP, indicating that CaGID1b.2 may play a prominent role in the placenta; in seed, CaGID1b.1 displayed the highest expression level at 0 DAP, while CaGID1b.2 had the highest fold change of 6.46 from 0 to 5 DAP. We also noticed that in all of the three tissues, the expression change of CaGID1c was milder than that of CaGID1b.1 and CaGID1b.2. The fold change data were shown in Appendix A. Therefore, it is inferred that CaGID1b.1 and CaGID1b.2 play more important role than CaGID1c during fruit development, including pericarp, placenta and seed.

Comparing the relative expression fold changes of the CaGID1b.1 and CaGID1b.2 in all the three tissues from 0 to 10 DAP at the same developmental stages, the relative expression in pericarp changed most greatly with the exception of CaGID1b.2 in placenta higher than pericarp from 0 to 5 DAP. Therefore, during most of the developmental stages, CaGID1b.1 and CaGID1b.2 may exert the greatest function in pericarp growth.

2.3. Overexpression of CaGID1s and CaGAI in Arabidopsis

Transgenic Arabidopsis plants of CaGID1b.1, CaGID1b.2 and CaGID1c under gid1a gid1c background were taller than the double mutant gid1a gid1c. Among these transgenic plants CaGID1b.2/gid1a gid1c displayed the tallest, but shorter than the wild type one. The stem heights of CaGID1b.1/gid1a gid1c and CaGID1c/gid1a gid1c were similar, which were a little taller than the double mutant gid1a gid1c (Figs. 4 and 5). The transgenic plants of mutant rga-24/gai-t6 overexpressing CaGAI displayed shorter than rga-24/gai-t6 (Fig. 6), but taller than wild type. The partially resumed phenotype indicated that these genes used for transformation affect plant growth, and may have similar functions in stem elongation as AtGID1 and AtDELLA, respectively.

2.4. Interactions between CaGID1s and CaGAI

The presence of GA3 triggered interactions of CaGID1b.1/b.2 and CaGAI, while no interaction occurred when GA3 was absent; however, CaGID1c interacted with CaGAI in presence or absence of GA3 (Fig. 7-A). The results about interactions between CaGID1s and CaGAI were reconfirmed by

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**Fig. 3** Relative expression of CaGID1s and CaGAI in different Capsicum tissues at different developmental stages. 0, 5, 10, 50, the number of days after pollination.
bimolecular fluorescence complementation (BiFC) approach (Fig. 7-B). When 100 μmol L⁻¹ GA₃ was added to the infiltration solution, CaGAI interacted with CaGID1b.1, CaGID1b.2 and CaGID1c, respectively, and no fluorescence occurred in negative controls; when no exogenous GA was added during the infiltration, only the combination of CaGAI and CaGID1c triggered fluorescence, which showed the same interaction result as yeast two-hybrid (Y2H) assay.

From the results shown above, we inferred that CaGID1b.1 and CaGID1b.2 interact with CaGAI, respective-

3. Discussion

3.1. Regulation of fruit set in Capsicum by CaGID1s

ly in a GA-dependent manner, while the interaction between CaGID1c and CaGAI is GA-independent.

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3.1. Regulation of fruit set in Capsicum by CaGID1s

Successful completion of pollination and fertilization leads to changes in gibberellin biosynthesis and their subsequent
signal transduction in plant ovaries and fruits, thereby regulating fruit set and development (Gillaspy et al. 1993; Ben-Cheikh et al. 1997; Ozga et al. 2002, 2003; De Jong et al. 2009; Zhang et al. 2010). Here we also observed the expression changes of \( \text{CaGID1s} \) and \( \text{CaGAI} \), participating in gibberellin signaling, caused by pollination and fertilization.

From previous studies, it was found that the expression of \( \text{GID1} \) was negatively regulated by active GAs content through a feedback mechanism in order to maintain GA homeostasis (Sun and Gubler 2004; Griffiths et al. 2006; Hirano et al. 2008). In our study, the expression level of \( \text{CaGID1s} \) was upregulated in placenta and seed of un-pollinated fruit with downregulation of active GAs content (data not shown), but the \( \text{CaGAI} \), whose expression was negatively affected by \( \text{CaGID1s} \), was also upregulated, which may be because that the upregulated expression of \( \text{CaGIDs} \) could not offset effect caused by the decline of GAs content.

It was also noticed that the \( \text{CaGID1s} \) expression level differed a lot between pollinated and un-pollinated fruit pericarp at 5 DAP or emasculation, while their GAs contents were similar (data not shown). Therefore, we infer that the expression level of \( \text{CaGID1s} \) may not only be regulated by GAs content, but also the pollination and fertilization signal may trigger some other pathways that affect \( \text{CaGID1s} \) expression in \( \text{Capsicum} \) pericarp.

### 3.2. The mild expression change of \( \text{CaGID1c} \) may be related to its GA-independent manner

In Y2H and BiFC assays, \( \text{CaGID1c} \) was shown to interact with \( \text{CaGAI} \) in a GA-independent manner, and displayed a higher interaction with deeper blue in Y2H assay (Fig. 7); therefore its affinity with \( \text{CaGAI} \) may not be affected by GAs, which was coincident with its relatively steady expression level compared with \( \text{CaGID1b.1} \) and \( \text{CaGID1b.2} \) during fruit set and development in \( \text{Capsicum} \).

In Yamamoto’s (2010) study, the 99th proline in OsGID1 was considered to be a key amino acid to the interaction manner, and according to the crystal structure provided by Shimada et al. (2008), proline is located in the loop region, which is not conserved. In \( \text{AtGID1a} \) and \( \text{AtGID1c} \), which interact with DELLa in a GA-dependent manner, there is a
proline in the loop of each protein. While no proline in the loop of AtGID1b, which is GA-independent, therefore the proline is considered to be a character for GA interaction manner. While in our study, we predicted the protein structure and a loop region existed in all of these three proteins — CaGID1b.1, CaGID1b.2 and CaGID1c (Appendix B), and there was no proline located in the loop of CaGID1b.1 and CaGID1b.2, while existing in CaGID1c (Appendix C). But according to Y2H and BiFC assays, the CaGID1b.1 and CaGID1b.2 were GA-dependent and CaGID1c was GA-independent, showing the opposite result to Arabidopsis. Therefore, some other factors may also affect the interaction manner except the proline located in the loop region.

3.3. The GID1s may not be identical among species in many aspects

According to phylogenetic analysis, GID1a and GID1c displayed similar in amino sequences, and were classified as a subfamily, while GID1b was classified as another subfamily with greater differences from GID1ac. From previous studies, there are three GID1 orthologs in Arabidopsis, grape, tomato and apple, respectively (El-Sharkawy et al. 2014). In Arabidopsis and grape, there are two members in GID1ac subfamily and one belonging to GID1b subfamily. While two members of tomato and apple belong to GID1b subfamily and one was classified among the GID1ac, and in our study the CaGID1s from Capsicum showed the same classification as tomato and apple.

There are three GID1s identified in Arabidopsis. They are AtGID1a, AtGID1b and AtGID1c. The single mutants developed normally, and the double mutants of gid1a gid1b and gid1b gid1c displayed similar plant height compared with wild type Arabidopsis, while double mutants gid1a gid1c displayed reduced stem height, indicating that subfamily GID1ac is of great importance in stem elongation in Arabidopsis (Griffiths et al. 2006; Iuchi et al. 2007). However, in our study CaGID1b.1 and CaGID1b.2 showed greater effect on stem elongation in Arabidopsis, suggesting a greater role of subfamily CaGID1b in stem elongation than that of subfamily CaGID1c. Therefore, the function of GID1s could not be classified only due to amino sequence.

The GA-dependent interaction between GID1 and DELLA protein had been recognized in some species, such as rice, Arabidopsis, plum and so on (Ueguchi-Tanaka et al. 2005; El-Sharkawy et al. 2014), while GA-independent manner also existed in plants, and some genes had been discovered in Arabidopsis, soybean and Brassica napus (Yamamoto et al. 2010). The GA-binding activity of AtGID1b is four times than that of AtGID1a and AtGID1c, and could interact with DELLA in presence or absence of active GA (Griffiths et al. 2006; Nakajima et al. 2006). While in our study, CaGID1c, belonging to another subfamily GID1ac, according to the phylogenetic analysis, could interact with CaGAI in presence or absence of active GA.

3.4. GA-dependent interaction between CaGID1 and CaGAI requires relatively higher active GAs content

The GID1s are different in sensitivity to GA, and high $K_a$ (association) value and low $K_d$ (dissociation) value lead to high sensitivity to GA (Yamamoto et al. 2010). In BiFC assay, although the tobacco leaves should contain endogenous active GAs, there was no fluorescence signal observed in the combination of CaGAI and CaGID1b.1/b.2 when GA was not added to the infiltration solution, this may be due to the scarcity of active GAs, which could not trigger GA-dependent interaction between CaGAI and CaGID1b.1/b.2. The similar phenomenon was also observed during the study of interaction between PsiGIDs and AtGAI/RGL1 (El-Sharkawy et al. 2014), whose interaction was in a GA-dependent manner.

4. Conclusion

During fruit set and development in Capsicum, three gibberellin receptor genes CaGID1b.1, CaGID1b.2, CaGID1c and one DELLA gene CaGAI were identified. According to gene expression analysis and ectopic expression, it is inferred that CaGID1s and CaGAI are involved in gibberellins signaling during fruit set and development, and have similar function to AtGID1 and AtGAI, and CaGID1b.2 displayed the greatest effect among CaGID1b.1, CaGID1b.2 and CaGID1c. Y2H and BiFC assays indicated that CaGID1b.1 and CaGID1b.2 interact with CaGAI in a GA-dependent manner, while CaGID1c interacts with CaGAI in a GA-independent manner.

5. Materials and methods

5.1. Plant materials and growth conditions

A pepper inbred line 0819 (Capsicum annuum L.) was planted in a greenhouse during August 2012 to February 2013 in Beijing. Ovaries or fruits at 0, 5, 10, 50 days after pollination (DAP) and 5 days after anthesis with non-pollination (DAA-NP) were collected. After removal of floral organs, pericarp (carpel), seeds (ovules) and placenta were carefully separated by a scalpel. All samples were frozen in liquid nitrogen and stored at −80°C before use.

The Arabidopsis mutants gid1a gid1c (Arabidopsis thaliana, ecotype Columbia), rga-24/gai-t6 (Arabidopsis thaliana, ecotype Landsberg) and their wild type plant seeds were provided by Prof. Ren Huazhong, China Agricultural University. The mutant of gid1a gid1c showed a dwarf phenotype compared with wild type, and rga-24/gai-t6 displayed...
taller than wild type.

The *Arabidopsis* plants were grown under 16:8 h and 23:17°C in day:night conditions. Tobacco (*Nicotiana benthamiana*) plants were grown in 16:8 h and 24:17°C in day:night conditions.

## 5.2. Gene identification, cloning and sequence analysis

GID1 protein sequences in *Arabidopsis thaliana*, *Solanum lycopersicum* and some other species (Fig. 1) obtained from the National Center for Biotechnology Information, China (NCBI, http://www.ncbi.nlm.nih.gov) were used to blast against the proteins of Zunla-1 (downloaded from http://peppersequence.genomics.cn) by BLASTP on local computer, and then blast the top hits proteins of Zunla-1 against the nr (non-redundant) database in NCBI to confirm their putative function by the topprest hit annotation. The remained proteins with topprest hit “GID1” were considered to be CaGID1s. DELLA protein was filtered by three conserved domains: DELLA, VHYNP and GRAS. Protein alignment was carried out by ClustalW in MEGAS.5.05 software. Phylogenetic analysis was performed by MEGAS.5.05 using neighbor-joining method. The boxes in the alignment analysis were drawn on the web site Boxshade (http://www.ch.embnet.org/software/BOX_form.html). The prediction of protein structure was carried out by Phyre2 (http://www.sbg.bio.ic.ac.uk/servers/phyre2/html). Full-length cDNA sequences of CaGID1s and CaGAI were PCR-amplified from a cDNA library of ovaries (0 DAP) and fruits (5 DAP) using specific primers in Appendix D.

## 5.3. RNA isolation and gene expression analysis

The RNA isolation and cDNA synthesis were performed using SV Total RNA Isolation System Kit (Promega, Madison, USA) and GoScript™ Reverse Transcription System Kit (Promega, Madison, USA) according to the manufacturer’s instruction. Gene expression analysis was performed by qRT-PCR with the LightCycler®480 SYBR Green I Master on a LightCycler®480 II (Roche, Basel, Switzerland) real-time PCR system. The *Capsicum* actin gene (*CaAct*, GU39766.1) was used as reference gene in *Capsicum* gene expression. The qRT-PCR was performed with two to three biological repeats and each reaction was repeated two to three times. All primers were shown in Appendix D.

## 5.4. Plant transformation

Full-length cDNA sequences of *CaGID1b.1*, *CaGID1b.2*, *CaGID1c* and *CaGAI* were introduced into *BamHI* site of pCAMBIA2300-35S vector (provided by Prof. Cui Xia, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences) using In-Fusion Cloning Kit (Clontech, Dalian, China) according to the manufacturer’s instruction, respectively. The constructed vectors were introduced into *Agrobacterium tumefaciens* (C58) by freeze-thaw method (Peng 2006). Floral dip method (Clough and Bent 1998) was used for *Arabidopsis* plants transformation.

## 5.5. Yeast two-hybrid assay (Y2H)

Full-length cDNA sequences of *CaGID1b.1*, *CaGID1b.2* and *CaGID1c* were introduced into the *BamHI* site of the pGBK77 bait vector, respectively, and *CaGAI* was introduced into the *BamHI* site of the pGAD77 prey vector. Both of the vectors were provided by Dr. Cui Xia, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences. The constructed vectors were transformed into AH109 strain yeast in pair using Yeastmaker™ Yeast Transformation System 2 (Clontech, Dalian, China) for yeast transformation according to the manufacturer’s instruction. Resuspended yeast of 5 microliters were spread on SD-Ade-Trp-His-Leu medium containing X-a-gal in the presence or absence of 100 μmol L⁻¹ GA₃ and grown at 30°C. Each assay repeated at least three times.

## 5.6. Bimolecular fluorescence complementation assay (BiFC)

Full-length cDNA sequences of *CaGID1b.1*, *CaGID1b.2* and *CaGID1c* were introduced into the *BamHI* site of the vector pSPYNE-35S, and *CaGAI* was introduced into the *BamHI* site of the pSPYCE-35S vector (Walter et al. 2004). The vectors pSPYNE-35S and pSPYCE-35S were provided by Prof. Zhang Xiaolan, China Agricultural University. The constructed plasmids were introduced into *Agrobacterium tumefaciens* (GV3101) by freeze-thaw method (Peng 2006), respectively. The transfection of tobacco mainly followed Waadt’s (2008) protocol in the presence or absence of 100 μmol L⁻¹ GA₃. Lower epidermis of infiltrated leaves was observed with a confocal laser scanning microscope after 2 days of infiltration.

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Appendix associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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