Characterization of stipe elongation of the mushroom *Coprinopsis cinerea*

Wenming Zhang, Xiuxiu Wu, Yajun Zhou, Zhonghua Liu, Wen Zhang, Xin Niu, Yan Zhao, Siyu Pei, Yang Zhao and Sheng Yuan

Jiangsu Key Laboratory for Microbes and Microbial Functional Genomics, Jiangsu Engineering and Technology Research Center for Industrialization of Microbial Resources, College of Life Science, Nanjing Normal University, Nanjing 210023, PR China

Previously, we observed an acid-induced short-term wall extension in *Flammulina velutipes* apical stipes during a 15 min period after a change from a neutral to an acidic pH. This acid-induced stipe wall extension was eliminated by heating and reconstituted by a snail expansin-like protein, although we failed to isolate any endogenous expansin-like protein from *F. velutipes* because of its limited 1 mm fast elongation region. In this study, we report that *Coprinopsis cinerea* stipes possess a 9 mm fast elongation apical region, which is suitable as a model material for wall extension studies. The elongating apical stipe showed two phases of acid-induced wall extension, an initial quick short-term wall extension during the first 15 min and a slower, gradually decaying long-term wall extension over the subsequent 2 h. After heating or protein inactivation pretreatment, apical stipes lost the long-term wall extension, retaining a slower short-term wall extension, which was reconstituted by an expansin-like snail protein. In contrast, the non-elongating basal stipes showed only a weaker short-term wall extension. We propose that the long-term wall extension is a protein-mediated process involved in stipe elongation, whereas the short-term wall extension is a non-protein mediated process not involved in stipe elongation.

**INTRODUCTION**

One of the characteristics of the development of mushroom fruit bodies is stipe elongation growth (Craig et al., 1977; Gruen, 1963; Voisey, 2010), which primarily results from manifold cell elongation (Gooday, 1985; Kamada & Takemaru, 1977; Shioya et al., 2013) rather than cell division as in the intercalary extension of hyphae of endophytic fungi of the genus *Epichloë* in mutualistic symbiosis with Pooidae grasses (Christensen et al., 2008). Because the stipe cell is surrounded by a wall layer that acts similarly to a straitjacket to constrain and shape the cell, cell elongation requires the irreversible extension of the pre-existing wall to create space for the enlarging protoplast (Bartnicki-Garcia, 1999; Kamada, 1991). Cell elongation of the stipe was proposed because of the enzymic hydrolysis of matrix polysaccharides in cell walls, which changes the wall mechanical properties and, therefore provides the wall plasticity (Kamada et al., 1982, 1985, 1991). However, Mol et al. (1990) proposed that cell elongation of the stipe resulted from the creeping of polymers due to the continuous breakage and reformation of hydrogen bonds among the glucan chains by stress from turgor pressure and by passive orientation of the chitin chains, rather than hydrolysis by lytic enzymes. Recently, we purified an expansin-like protein from snail stomach juice, which could reconstitute heat-inactivated *Flammulina velutipes* stipe wall extension without hydrolytic activity at an acidic pH (Fang et al., 2014). Our results do not support the previous hypotheses that the hydrolysis of wall polymers by enzymes (Kamada et al., 1991) or the disruption of the hydrogen bonding of wall polymers, only by the stress of turgor pressure (Mol et al., 1990), directly corresponds to stipe wall extension. Instead, we proposed that, similar to higher plant wall extension (Cosgrove, 2000, 2005, Wang et al., 2013), stipe wall extension may be mediated by some endogenous expansin-like proteins that facilitate polymer slippage for stipe wall extension under stress by disrupting hydrogen bonding among wall polymers. However, we failed to isolate and purify any endogenous expansin-like protein from *F. velutipes* because the fast elongation region is limited to the 1 mm apical region of the stipe, which may contain a much lower amount of expansin-like protein (if this protein exists) (Fang et al., 2014). In addition, the acid wall extension profile of *F. velutipes* stipe did not exhibit a classic long-term phase similar to that in plant cell walls (Cosgrove, 1989).

In this study, we report that *Coprinopsis cinerea* stipes have approximately 9 mm fast elongation growth regions in 27 mm growing fruit bodies, which are only enclosed by the cap. This large growth region is an advantage that makes *C. cinerea* convenient for the characterization of stipe wall extension. As a result, the acid-induced wall extension in *C. cinerea* stipes is divided into two phases, a short-term
fast wall extension and a long-term wall extension continuing over the subsequent 2 h. The former is a heat-insensitive process, whereas the latter is a heat-sensitive protein-mediated process involved in stipe elongation growth.

**METHODS**

**Strain and culture.** *C. cinerea* ATCC 56838 was purchased from the American Type Culture Collection (ATCC). For Petri dish cultivation, an agar block with mycelium was inoculated on the centre of potato dextrose agar plus yeast (PDAY) medium agar (300 g diced potatoes, 20 g glucose, 15 g agar, 5 g yeast extract and 1 l distilled water) in Petri dishes 9 cm in diameter and incubated at 28 °C under conditions described by Kamada et al. (1982) until appropriate lengths of fruit bodies were formed.

**Measurement of elongation growth of the developing stipe.** Approximately one-third of a cap was vertically removed from an approximately 27 mm-long fruit body of *C. cinerea* grown on PDAY medium in Petri dishes to expose the apical part of the stipe enclosed by the cap. The entire stipe was divided into different regions from the apex to the base at 1 mm intervals with a marker pen. The change in length of each division was measured at 1 h intervals.

**Fig. 1.** The distribution of elongation growth along the length of the developing stipe of *C. cinerea*. (a) Photographs of the elongation growth of a stipe. To determine the distribution of elongation growth along the length of the developing stipe, approximately one-third of the cap was vertically removed from a 27 mm fruit body grown on agar medium in a Petri dish to expose the upper part of the stipe enclosed by the cap. The entire stipe was divided into different regions from the apex to the base at 1 mm intervals with a marker pen. The change in length of each division was measured at 1 h intervals. (b) Summary of the elongation growth of the stipes (*n*=9). Coloured segments represent different stipe growth regions from 1 at the apex and 20 at the base.
Fig. 2. The pH dependence of wall extension of the apical divisions of *C. cinerea* stipes. First, 1–9 mm apical fragments from 27 mm-long *C. cinerea* stipes were frozen, thawed, abraded, compressed and then clamped to an extensometer with a loading force of 3 g. The 5 mm region of the stipe fragments between the clamps was suspended in a 150 µL bathing solution in a cuvette in an extensometer for the measurement of wall extension. (a) Apical stipe fragments were incubated in 50 mM sodium acetate (pH 4.0–6.0) or in 50 mM HEPES (pH 7.0) bathing solution for 150 min. (b) Apical stipe fragments were initially suspended in 50 mM HEPES (pH 6.8) bathing solution for 30 min, after which the bathing solution was replaced by 50 mM sodium acetate (pH 4.0–6.0) or 50 mM HEPES (pH 7.0) solution and incubated for the next 120 min. Arrows indicate when the bathing solution was replaced. Curves [a(i)], [a(ii)], [b(i)] and [b(ii)] are the means of more than three independent experiments (n=9). Histograms [a(iii)] and [b(iii)] are the means ± SEM (n=9), and wall extension activity (% h⁻¹) was calculated as (final rate–initial rate)/wall length (McQueen-Mason *et al.*, 1992).
by the cap. The entire stipe was divided into different regions from the apex to the base at 1 mm intervals with a marker pen. Then, the marked fruit bodies grew continuously in the agar medium, and the change in length of each division of the stipe was measured every hour for 7 h (Cox & Niederpruem, 1975).

**Isolation and purification of the snail expansin-like protein.** The expansin-like protein was isolated and purified from the stomach juice of the snail *Helix aspersa* as described by Fang et al. (2014).

**Measurement of stipe wall extension.** Approximately 9 mm apical stipe segments removed from enclosed caps were used for all measurements of wall extension, except for the determination of wall extension for different stipe regions, in which 9 mm lengths of segments from different regions were measured as indicated in the text. The above-mentioned fresh segments of *C. cinerea* stipes were abraded with carborundum, frozen at −20 °C for approximately 24 h or more and used within 1 week for measurements.

Before measurement, frozen segments were thawed at room temperature and bisected vertically into halves with a blade. After each stipe half was compressed between two glass slides, the sample was clamped in a constant-load extensometer under a constant tension of 3 g, and the specimen length between the clamps was 5 mm (Cosgrove, 1989). Tissues between clamps were incubated in 0.15 ml bathing solution, and changes in specimen length were recorded at 30 s intervals using a linear voltage displacement transducer in the extensometer (McQueen-Mason et al., 1992).

For the measurement of wall extension, stipe segments were first incubated in 0.15 ml initial bath solution in cuvettes in an extensometer for 30 min, after which the bathing solution in each cuvette was replaced by the indicated bath solution as described in the text for another 120 min of incubation.

For the inactivation of endogenous wall extension activity (Cosgrove, 1989), 9 mm apical stipe sections were incubated in 2 mg ml$^{-1}$ Pronase protease (Sigma, P5147) in 50 mM sodium acetate (pH 5.0) containing 1 mM DTT or in 0.2 % SDS for 1 h at 30 °C or boiled in water for 2 min or in methanol for 5 min prior to the measurements, and then they were clamped to an extensometer for wall extension analysis.

**RESULTS**

**Profile of elongation growth of the developing fruit body stipe**

To investigate the elongation growth of *C. cinerea* stipes, the 7 h growth course of an approximately 27 mm long young fruit body was recorded (Fig. 1a). During the

![Graph](image-url)

**Fig. 3.** Region dependence of wall extension of *C. cinerea* stipes. For this analysis, 1–9 mm apical, 10–18 mm medial or 19–27 mm basal fragments from 27 mm-long *C. cinerea* stipes were treated and subjected to extensometric analysis as in Fig. 2. (a) Stipe fragments were incubated in 50 mM sodium acetate (pH 4.5) bathing solution for 150 min. (b) Stipe fragments were initially suspended in 50 mM HEPES (pH 6.8) bathing solution for 30 min, after which the bathing solution was replaced by 50 mM sodium acetate (pH 4.5) solution with incubation for the next 120 min. Arrows indicate when the bathing solution was replaced. The curves are the means of more than three independent experiments ($n=9$).
development of the fruit body, the apical and median regions of the stipe elongated. The 1–9 mm apical region enclosed by the cap was the region most extended and kept a steady elongation rate, by approximately onefold every 2 h in the first 4 h after marking. The 10–18 mm median region below the cap also exhibited considerable elongation, by approximately 0.6-fold every 2 h in the first 4 h after marking, after which the region no longer elongated. In contrast, the 21–27 mm swollen base region did not elongate during fruit body development, and the 19–20 mm region, far from the apex, only slightly elongated during the first 1 h and then elongation ceased (Fig. 1b). 

The profile of C. cinerea stipe wall extension

For the characterization of stipe wall extension, this was determined by the extensometer. As shown in Fig. 2(a), the 1–9 mm apical divisions enclosed by the cap of 27 mm-long fruit body stipes could be extended by an exogenous strain force. The stipe wall extension appeared to be pH dependent, i.e. the wall extension activity of the stipe at pH 7.0 was only 1.03 % h⁻¹, whereas the extension activity increased with a decrease in pH and reached 2.03 % h⁻¹ at pH 4.5, which was twofold the extension activity at pH 7.0 (Fig. 2a). Apical stipe wall extension at pH 4.5 appeared to have a higher initial rate (3.34 μm min⁻¹), quickly decreased to a basal level (1.4 μm min⁻¹) during the first 15 min and then this extension exhibited a slower, decaying profile during the following 120 min (Fig. 3a). Stipe wall extension was dependent on the stipe region (Fig. 3a), i.e. the 10–18 mm median region of the 27 mm fruit body stipe showed a lower wall extension activity compared with that of the 1–9 mm apical region, whereas the 19–27 mm basal region only showed weaker wall extension activity at pH 4.5.

When the 1–9 mm apical sections of the 27 mm fruit body stipes were first incubated in a neutral bath solution (50 mM HEPES, pH 6.8) for 30 min and then changed to an acidic bath solution (pH 4.5) for 30 min and then changed to an acidic bath solution (pH 4.5), an apparent, strong and rapid wall extension was immediately induced (Fig. 3a). Wall length change, (b) wall extension rate, (c) wall extension activity. Arrows indicate when the bathing solution was replaced. The curves are the means of more than three independent experiments (n=9). The histogram shows the means ± SEM (n=9); wall extension activity (% h⁻¹) was calculated as (final rate – initial rate)/wall length (McQueen-Mason et al., 1992).
2b). The optimum pH for stipe wall extension was 4.5. Similarly, the acid-induced rapid wall extension of the apical stipe showed a higher initial rate that quickly decreased to an essential level within approximately 15 min, which was designated short-term wall extension. Then the wall extension entered a slower decaying period over the course of approximately 1–2 h, which was designated long-term wall extension (Fig. 3b). The 10–18 mm median divisions of these stipes showed a similar wall extension profile but at a lower extension rate (Fig. 3b). Notably, although 19–27 mm basal divisions showed a faint acid-induced short-term wall extension, this extension rate quickly decreased to zero, i.e. the long-term wall extension disappeared (Fig. 3b).

Stipe wall extension of *C. cinerea* includes a heat-sensitive wall extension and a heat-insensitive wall extension

Interestingly, the experiments demonstrate that heat treatment in boiling water or in boiling methanol eliminated 66.38% and 68.77%, respectively, of the acid-induced short-term wall extension activity in apical stipes during the first 15 min after changing to an acidic bath solution (pH 4.5) from a neutral bath solution (pH 6.8) compared with untreated specimens and completely eliminated the acid-induced long-term wall extension of apical stipes (Fig. 4).

This heat-insensitive wall extension of heated stipes was dependent on the pH of the bath solution. No heat-insensitive wall extension was observed when the heated stipes were shifted from a neutral bath solution to a pH 7.0 bath solution (Fig. 5). This heat-insensitive wall extension activity of heated stipes was higher in the apical region enclosed by the cap than that in the median region, and was extremely faint in the basal region (Fig. 6).

**Fig. 5.** pH dependence of heat-insensitive wall extension of heated stipes of *C. cinerea*. Nine-millimetre apical fragments from 27 mm-long *C. cinerea* stipes were prepared, heated and subsequently subjected to extensometrical analysis as in Fig. 3(b), except that the initial bathing solution was replaced with 50 mM sodium acetate (pH 4.0–6.0) or HEPES (pH 7.0). (a) Wall length change, (b) wall extension rate. Arrows indicate the time when the bathing solution was replaced.

**Fig. 6.** Heat-insensitive wall extension activity in different regions of *C. cinerea* stipes. Nine-millimetre apical, median or basal fragments from 27 mm-long *C. cinerea* stipes were prepared, heated and subsequently subjected to extensometrical analysis as in Fig. 3(b). (a) Wall length change, (b) wall extension rate. Arrows indicate when the bathing solution was replaced.
Heat-sensitive wall extension of stipes is a protein-mediated process

To test whether heat-sensitive wall extension is a protein-mediated process, stipe walls were treated with either protein denaturants or hydrodases to denature or inactivate the activity of the potential endogenous wall proteins before determination. Experiments showed that, similar to boiling in water or methanol, SDS or Pronase pretreatment eliminated 59.90% and 58.53%, respectively, of the acid-induced short-term wall extension activity and all of the acid-induced long-term wall extension of apical stipes compared with untreated stipe walls after changing to an acidic bath solution (pH 4.5) from a neutral bath solution (pH 6.8) (Fig. 4).

Previously, we reported that an expansin-like protein isolated from snails induces wall extension of heat-inactivated stipes of *F. velutipes* fruit bodies (Fang et al., 2014). In this study, we report that this expansin-like snail protein reconstituted the heat-sensitive, long-term wall extension in *C. cinerea* stipes (Fig. 7). The exposure of heated stipes to 10 µg ml⁻¹ expansin-like snail protein resulted in maximum wall extension rates of 1.28 µm min⁻¹, 0.89 µm min⁻¹ and 0.25 µm min⁻¹ in the apical, median and basal regions of *C. cinerea* stipes, respectively, indicating that different regions of *C. cinerea* stipes have different susceptibilities to this snail protein (Zhao et al., 2008). Furthermore, the wall extension induced in heated stipes of *C. cinerea* by the expansin-like snail protein was dependent on acidic pH conditions (Fig. 8) and protein concentration (Fig. 9).

**DISCUSSION**

One of the aims of this study was to discover mushrooms with a longer elongating region in the stipe. Eilers (1974) reported that the upper 2/3 of the stipe in *C. cinerea* was responsible for 80–90% of the total growth, with the basal 1/3 contributing only approximately 10%. Furthermore, our research precisely determined that the apical 9 mm-long region enclosed by the stipe cap elongates most, the middle 9 mm-long region shows considerable elongation, but the 9 mm basal region essentially does not elongate during the development of the 27 mm-long young *C. cinerea* fruit body. We presume that the cap produces a growth regulator that governs stipe elongation (Eilers, 1974; Haindl & Monzer, 1994); therefore, the region enclosed by the cap more easily obtains the growth regulator for maximal elongation compared with other regions. Consistent with this result, the cell wall extension activity measured by extensometry showed a similar distribution pattern for the stipe from the apical region to the basal region. Because stipe specimens were frozen, thawed and compressed to remove the cell sap and eliminate turgor pressure before loading into the extensometer (Cosgrove, 1989), synthetic processes (Bowman & Free, 2006; Kamada et al., 1991; Mol et al., 1990; Shioya et al., 2013; Voisey, 2010) and endogenous turgor pressure (Mol et al., 1990; Money & Ravishankar, 2005) did not contribute to wall extension during measurement. Therefore, wall extension activity reflects wall properties (Cosgrove, 1993). Our results suggest that fruit bodies of *C. cinerea* have the following advantages as a model material to study stipe elongation growth. (1) The fastest elongating region of the stipe is 9 mm in length, which is suitable for the sample length requirement of the extensometer. (2) The stipes diameter of *C. cinerea* fruit bodies cultivated in Petri dishes is sufficiently slender so that half of the vertically bisected stipe is suitable for the sample diameter requirement of the extensometer. (3) The apical region of *C. cinerea* stipes has high wall extension activity, approximately 2 % h⁻¹, which is favourable for the study of the characteristics of cell wall extension.

In a previous experiment, we only observed a short-term acid-induced wall extension in *F. velutipes* apical stipes during a 15 min period after switching from a neutral to an acidic pH. However, in plant cells, acid-induced wall
Fig. 8. Effect of pH on expansin-like snail protein-reconstituted heat-inactivated wall extension of C. cinerea stipes. Nine-millimetre apical stipe fragments were prepared, heated and subsequently subjected to extensometrical analysis as in Fig. 3(b), except that the initial 50 mM sodium acetate (pH 4.5) bathing solution was replaced by 50 mM sodium acetate (pH 4.0–6.0) or 50 mM HEPES (pH 7.0) containing 10 µg ml⁻¹ expansin-like snail protein. For comparison, the treated specimens were initially suspended in 50 mM HEPES (pH 6.8) as a control. (a) Wall length change, (b) wall extension rate. Arrows indicate when the bathing solution was replaced.

Fig. 9. Concentration dependence of expansin-like snail protein-reconstituted heat-sensitive wall extension of C. cinerea stipes. Nine-millimetre apical stipe fragments were prepared, heated and subsequently subjected to extensometrical analysis as in Fig. 3(b), except that the initial bathing solution was replaced with 50 mM sodium acetate (pH 4.5) containing varying amounts of expansin-like snail protein. Controls were not exposed to snail protein. (a) Wall length change, (b) wall extension rate. Arrows indicate when the bathing solution was replaced.
extension involves two phases, a short-term wall extension phase (e.g. 0–5 min) and a longer-term wall extension phase (5 min to several hours) (Cosgrove, 1993; Shieh & Cosgrove, 1998). We attributed this single short-term acid-induced wall extension in *F. velutipes* to a limited 1 mm fast-extension apical region in its stipe because two 2 mm ends of an approximately 10 mm-long stipe specimen had to be clamped by the extensometer, and only the low wall-extension activity of the subapical region between clamps was determined (Fang et al., 2014). Utilizing the benefit of *C. cinerea* stipes that have widespread extension of apical regions, we explored two phases of acid-induced wall extension in *C. cinerea* stipes, i.e. a short-term fast wall extension during the first 15 min and a long-term wall extension over the subsequent 2 h.

Acid-induced long-term wall extension in plant cell walls was considered a protein-mediated process (Cosgrove, 1989, 1993). This idea led Cosgrove and his colleagues to finally discover expansin proteins, which mediate acid wall extension without hydrolytic activity, from a series of plant walls (Cosgrove et al., 1997; Li, Z. C. et al., 1993; Li, L. C. et al., 2003; McQueen-Mason et al., 1992; Sampedro & Cosgrove, 2005). This study demonstrates that treating walls of *C. cinerea* with heating, protease or protein denaturants eliminated the acid-induced long-term wall extension activity of *C. cinerea* stipes exactly as seen in plant cell walls (Cosgrove, 1989), implying that this acid-induced stipe wall long-term extension is a protein-mediated process. Previously, we reported that an expansin-like snail protein lacking hydrolytic activity could reconstitute the heat-inactivated wall extension of *F. velutipes* stipes (Fang et al., 2014). In the present study, we proved that this snail protein could also reconstitute the heat-inactivated wall extension of *C. cinerea* stipes in a concentration-dependent and acidic pH-dependent manner. Overall, these results indicate that some endogenous cell wall protein(s) must act as a catalyst for stipe wall extension. Definite proof of an expansin-like protein mechanism will require the extraction and reconstitution of the relevant proteins from stipe walls.

Notably, denaturation or inactivation treatments eliminated almost all long-term acid-induced wall extension activity, whereas these treatments only eliminated approximately 50% of the acid-induced short-term wall extension activity of apical stipes. Because the basal sections of stipes lost their elongation capacity, but still displayed a weaker acid-induced short-term wall extension activity, we conclude that the weaker, non-protein-mediated, acid-induced short-term extension in stipe walls is not involved in stipe wall elongation growth. Thus far, non-protein-mediated short-term extension has not been reported in plant cell walls, and heated or denatured plant walls did not display any remaining short-term wall extension (Cosgrove, 1998; McQueen-Mason et al., 1992; Zhao et al., 2008). The non-protein-mediated, heat-insensitive wall extension of stipes may be related to their specific chitin component in the stipe wall structure, which is lacking in plant cell walls (Bowman & Free, 2006; Ruiz-Herrera & Ortiz-Castellanos, 2010). The hydrogen atom on the amine group of N-acetylglucosamine of one chitin chain can form an N-H...O hydrogen bond with the oxygen atom on the carbonyl group of another neighbouring chitin chain. The bond energy of this type of hydrogen bond is only 2.1 kcal mol\(^{-1}\), which is approximately half of that of the O-H...O hydrogen bond between the hydrogen atom on the hydroxyl group of one glucan chain and the oxygen atom on the carbonyl group of a neighbouring glucan chain (Ramakrishnan & Prasad, 1972). Therefore, it is possible that the hydrogen bonds between chitin chains are easily disassociated in an acidic solution. Indeed, Hejazi & Amiji (2003) reported that hydrogen bonds dissociate at low pH due to the protonation of the amine groups of chitosan, which is the deacetylated form of chitin. Thus, protonation at a low pH may also apply to the dissociation of hydrogen bonds between chitin chains with the partial deacetylated form in elongating stipe walls (Mol & Wessels, 1990). However, acidic pH alone is not enough to disrupt the O-H...O hydrogen bonds between glucan chains because of the high bond energy (5.4 kcal mol\(^{-1}\)). As shown in plant cell walls, protonation may reduce the free energy of hydrogen bond dissociation and render the bonds sensitive to protein function. Perhaps this can explain why acid induction is required for expansin-like protein-reconstituted wall extension in plants (Cosgrove 2000, 2005) or in basidiomycetes (Fang et al., 2014). Regarding the fact that this heat-insensitive short-term wall extension shows a gradient that is high in the apical and low in the basal region, this gradient may indicate that some other cross-links, in addition to hydrogen bonds, may occur between polysaccharides in stipe walls along with cell wall maturation during the development of fruit bodies (Cabib & Arroyo, 2013; Zhao et al., 2008).

**ACKNOWLEDGEMENTS**

This work was supported by the National Natural Science Foundation of China (No. 31170028), the Priority Academic Development Program of Jiangsu Higher Education Institutions and the Scientific Innovation Research Program of Graduates at Nanjing Normal University (CXLX13-384).

**REFERENCES**


Edited by: I. V. der Klei