A small molecule inhibits cell elongation by modulating cell wall polysaccharide composition in Arabidopsis

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\textbf{Abstract}

The plant primary cell wall is comprised of pectin, cellulose and hemicelluloses, whose dynamic interactions play essential roles in plant cell elongation. Through a chemical genetics screening, we identified a small molecule, named cell wall modulator (CWM), which disrupted cell growth and deformed cell shape in etiolated Arabidopsis hypocotyl. A pectin defective mutant qua2, identified from screening an Arabidopsis EMS mutant library, showed a reduced sensitivity to CWM treatment. On the other hand, pectinase treatment suppressed the CWM induced phenotype. Furthermore, cellulose content was decreased in response to CWM treatment, while the cellulose synthesis mutants irx1 and irx2 were hypersensitive to CWM. Together, the study identified a small molecule CWM that induced a modification of the cell wall in elongating cells, likely through interfering with pectin modification. This molecule may be used as a tool to study cell wall remodeling during plant growth.

1. Introduction

The primary cell wall plays a variety of essential roles in plant growth, such as shaping cells, controlling cell growth, determining cell differentiation and sensing external stimuli (Bidhendi and Geitmann, 2016; Cosgrove, 2018; Koegst et al., 2010; Vosmeur and Hofte, 2016). The primary cell wall is structured with various polysaccharides (including cellulose, hemicelluloses and pectins) and wall proteins (Cosgrove and Jarvis, 2012). Pectic polysaccharides are mainly composed of \(\alpha\)-galacturonic acid (D-Gal-A) and are increasingly considered as important regulators of cell wall construction and plant growth (Anderson, 2016; Biswal et al., 2018; Hocq et al., 2017). Homogalacturonan (HG) is the most abundant pectin polysaccharide, which is synthesized in the Golgi apparatus in a methylesterified form (Atmodjo et al., 2013). After the methylated HG is deposited into the cell wall, demethylesterification and other modifications are carried out by a battery of enzymes associated with cell growth and responding to external stimuli (Altartouri et al., 2019; Haas et al., 2020; Peaucelle et al., 2015). The enzymes for pectin modification include pectin methyl-esterases (PMEs) (Willats et al., 2001), PME inhibitors (PMEI), polygalacturonases (PGs), and pectate lyases-like (PLLs) (Senechal et al., 2014). PGs and PLLs cleave the \(\alpha\)-1,4 bond of D-Gal-A units mainly from the HG backbone through hydrolysis or \(\beta\)-elimination, respectively (Senechal et al., 2014). Pectin modification in the cell wall is closely related to wall elasticity and growth (Peaucelle et al., 2012, 2015). Mutations in the putative pectin methyltransferase QUASIMODO2 (QUA2) reduce pectin content, cell adhesion and etiolated hypocotyl growth (Krupkova et al., 2007; Mouille et al., 2007). Overexpression of \textit{PECTIN METHYLESTERASE 5} (PM5) leads to decrease of pectin methylesterification and enhancement of primordia cell growth (Peaucelle et al., 2011). Transcriptomic and genetic analyses reveals that pectin de-methylesterification and degradation are important for initiation of the growth acceleration in the etiolated hypocotyls (Pelletier et al., 2010). Mutation of the PG gene \textit{polygalacturonase involved in expansion 1} (pgx1) inhibits hypocotyl elongation (Xiao et al., 2014). Thus, pectin methylesterification and demethylesterification play an important role in plant cell growth.

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\textbf{Abbreviations:} HG, homogalacturonan; RG-I, rhamnogalacturonan-I; RG-II, rhamnogalacturonan-II; CSCs, cellulose synthase complexes; CESA, cellulose synthase; DE\%, degree of methylesterification.

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In the cell wall, pectin, cellulose and hemicelluloses form an intricate matrix structure (Dick-Pérez et al., 2011; Lin et al., 2016; Wang et al., 2015). Cellulose microfibrils, which are synthesized by large cellulose synthase complexes (CSCs), are highly ordered crystallites formed by β-1,4-linked glucan chains packed in parallel through hydrogen bonds and van der Waals interactions (Kumar and Turner, 2015; Taylor, 2008). CSCs move at constant rates in linear tracks that are aligned and coincident with cortical microtubules (Paredes et al., 2006), resulting in parallel alignment of cellulose microfibrils with cortical microtubules (Li et al., 2012). Growth symmetry breaking in hypocotyl cells is controlled by pectin modification, which takes place prior to cortical microtubule reorientation (Peaucelle et al., 2015). Interconnection of pectin and cellulose may play a role in cell growth (Wang et al., 2015). However, whether the change of pectin methylesterification is correlated to cellulose deposition during cell growth is yet to be tested.

Small molecules can modulate biological processes and are widely used to study various processes in plants in situations of gene redundancy (Hicks and Raikhel, 2012; Toth and van der Hoorn, 2010). For example, small molecules were used to study abscisic acid (ABA) signaling (Park et al., 2009), natural variation of Arabidopsis (Zhao et al., 2007), endomembrane trafficking (Robert et al., 2008), cellulose biosynthesis (Debout and Brabham, 2013; Desprez et al., 2002) and cell wall formation (Harris et al., 2012). Here we report identification of a small molecule CWM which displayed an activity in inhibiting cell elongation. Chemical and genetic analyses revealed that CWM interferes with pectin modification and cellulose biosynthesis in hypocotyls. CWM may be used as a chemical genetic tool for studying modification of the cell wall intricate composition in cell growth.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana (Columbia) was used as wild type (WT) and all the plants were grown in a phytotron with a light/dark cycle of 16/8 h at 22 °C. Seeds were surface-sterilized in 5% NaClO for 10 min, washed 5 times in sterile pure water, and sprayed on the petri dish plates containing 1/2 Murashige and Skoog medium (MS), 0.7% agar and 1% sucrose. Sucrose was removed from growth medium when the plates were used to treat plants with small molecules. For observation of hypocotyl elongation in dark, plates were placed at 4 °C for 3 days, then exposed to light for 1–4 h and transferred to dark for 3 days (22 °C) before hypocotyl was analyzed. Agrobacterium mediated transformation was carried out following the floral dip method (Clough and Bent, 1998).

2.2. Chemical genetic screening and small molecule treatment

Chemical genetic screening was performed as previously described, using a chemical library bought from Life Chemicals Inc (Li et al., 2017; Ye et al., 2016; Ye and Zhao, 2018). The 96-well plates of the small molecule library with 1 μL of 10 mM small molecule solution in each well were mixed with 35 μL MS and 64 μL 1% melted agar. After cooling down, each well was mixed with 15 mL sterilized Arabidopsis seeds in 0.1% agar solution. Seeds number and agar solution volume were regulated to ensure there are about 15 seeds in 20 μL agar solution. 20 μL agar solution containing about 15 seeds was added into each well of the 96 well plate. A total of 12,000 small molecules were screened. For small
molecule treatment, unless otherwise stated, the plates were kept in dark for 3 days for hypocotyl elongation. Etiolated hypocotyls were observed and photographed under a SZX16 dissecting microscope. The length of hypocotyls was determined using Image J software (Freeware, National Institute of Health). At least 15 hypocotyls were analyzed in each independent experiment, each experiment was repeated three times, and mean ± SE was calculated for each data set. Student’s t test was used in the statistical analysis. Chemicals and isoxaben (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. To examine the effects of pectinase along with the small molecule, the enzyme derived from the fungus *Aspergillus niger* (Sigma-Aldrich) was used (Yoneda et al., 2010).

### 2.3. Screening of CWM resistant mutants

For isolation of CWM resistant mutants (mutants with reduced sensitivities to CWM compared to WT), we performed a screening through *Arabidopsis* EMS mutant library according to the previous description (Li et al., 2017). *Arabidopsis* seeds were grown without light on the growth medium containing 1/2 MS, 0.7% agar and 20 μM CWM. The seedling phenotypes were examined under the microscope, and those with longer hypocotyls were considered as putative CWM resistant mutants, which were verified in the next generation. Using map-based cloning, the mutant gene was identified by the CWM resistant phenotype, followed with candidate genes DNA sequencing analysis. Molecular markers are from the Arabidopsis Mapping Platform (Hou et al., 2010). About 200 F2 seedlings with the CWM resistant phenotype were used for mapping.

### 2.4. Microscopy analysis and ruthenium red staining

For observation under a cryo-scanning electron microscope (JEOL-6360LV carry Quorum PP301OT), the *Arabidopsis* seedlings were treated by 20 μM CWM or DMSO, the hypocotyls of etiolated seedlings were directly frozen in liquid nitrogen and then sublimation for about 5 min, after that, the samples were sputter coated with platinum and observed at an accelerating voltage of 10 kV on a cold stage kept at –140 °C.

Ruthenium red staining was used to observe the changes in pectin esterifications as previously described (Lionetti, 2015). Pollen tubes were dipped in ruthenium red solution (0.5 μg/μL, diluted in sterile pure water) and subjected to vacuum infiltration for 10 min. Excess of dye was removed by several washes with water. The seedlings were observed under a SZX16 dissecting microscope.

### 2.5. Analysis of pectin and cellulose content

Content of pectin and cellulose was measured according to the previous study (Foster et al., 2010; Yoneda et al., 2010). Hypocotyls (~2 g) from 3-day dark-grown seedlings were collected by excision of the cotyledons with a razor blade, grinded in liquid nitrogen. Then the sample was successively washed with 70% (v/v) ethanol, chloroform/methanol (1:1 v/v), and acetone and used as alcohol-insoluble residue (AIR) for analysis. AIR was destarched using α-amylase (1 μg/mL) and pullulanase (10 units/mL) in 0.1 M sodium acetate buffer (pH 5.0), washed with water and acetone, and dried at 45 °C (Zhang et al., 2018).

For pectin analysis, about 2 mg cell wall AIR was boiled with 12.5 mM sodium tetraborate in concentrated sulfuric for 5 min, then incubated with 0.15% m-dihydroxydiphenyl reagent in 0.5 M sodium hydroxide for 2 h. The resulted uronic acid content was determined by detecting the absorption at 520 nm.

For cellulose analysis, about 2 mg of the AIR residue was incubated with 2 M trifluoroacetic acid at 121 °C for 90 min and then centrifuged. The resulted pellet was washed with water followed by acetone for 3 times and dried. 1 ml of updegraff reagent (acetic acid: nitric acid: water = 8:1:2, v/v) was added to the dry material and incubated at 100 °C for 30
min. Mixture was cooled at room temperature, centrifuged at 10,000 rpm for 10 min, and the pellet was washed with water followed by acetone for 3 times and dried again. The resulted material was reacted with 175 μL sulfuric acid (72%) at room temperature for 1 h for hydrolyzation, then added 825 μL sterile pure water, centrifuged at 12,000 rpm for 5 min, and the resulted glucose content in the supernatant was determined using colorimetric anthrone assay according to previous description (Zhang et al., 2018).

2.6. Plasmid construction and plant transformation

Plasmid construction and plant transformation were performed as previously described (Ye et al., 2016). For complementation of the qua2-3 mutant, the coding sequence (CDS) of QUA2 was PCR amplified and constructed to a pGWB6 vector and transformed to the qua2-3 mutant.

2.7. Accession numbers

QUA2 (AT1G78240); PMR6 (AT3G54920); IXR1/CESA3 (AT5G05170); IXR2/CESA6 (AT5G64740); CWM (F0778-0383).

3. Results

3.1. CWM inhibited cell elongation in Arabidopsis hypocotyls.

The etiolated hypocotyl is an ideal system to study cell elongation as it elongates quickly without cell division (Gendreau et al., 1997). To search for small molecules that affect cell growth, we screened a chemical library (containing 12,000 small molecules) using Arabidopsis hypocotyls (Li et al., 2017; Zhao et al., 2007). Columbia (Col-0) seeds were surface sterilized and grown on 1/2 MS mediums in 96 well plates. Each well contained one chemical at the concentration of 100 μM. Hypocotyl phenotypes were imaged after growth for 3 days without light. 44 chemicals were identified to show inhibition on hypocotyl elongation. Among them, the chemical, N-(2-(3,5-dimethylphenyl)-4,6-dihydro-2H-thieno [3,4-c] pyrazol-3-yl) cyclopropanecarboxamide (Fig. 1 A) inhibited hypocotyl elongation in the dark at a low concentration (IC₅₀ 5 μM) and the inhibition was saturated at 30 μM (Fig. 1 B, C; Supplementary Fig. S1). Some of hypocotyl cells were compressed and deformed with diminution of transparency at the surface (Supplementary Fig. S2). To further characterize the cell morphology, we conducted a cryo-scanning electron microscope analysis. The chemical caused the hypocotyl cells unable to normally elongate in a parallel alignment manner. The cells were reshaped, showing an irregularity in cell anisotropy (Fig. 1 D). The cell length was reduced by about 70% (Fig. 1 E). This chemical is named CWM (cell wall modulator) as it is found to modulate the composition of cell walls during cell growth.

To confirm the function of CWM and its bioactive groups, we further examined the effect of other 24 CWM analogs in the chemical library. Four of the analogs also caused hypocotyls with shortened and spotted cells, similar phenotypes to those caused by CWM (Fig. 2 A, Supplementary Fig. S3). This confirmed our primary screening, of which CWM inhibited elongation of etiolated hypocotyls. The bioactive analogs share a similar chemical structure that is scaffolded with amide group, thieno-pyrazol group and benzene ring (Fig. 2 B, Table S2), which may be required for the chemical activity.
3.2. qua2-3 mutant showed a reduced CWM sensitivity.

To find how CWM inhibited cell elongation, we carried out a genetic screening against ethyl methane sulfonate (EMS) mutagenized M2 seeds of Col-0. In screening of approximate 13,000 lines, 94 putative mutants with CWM reduced sensitivity were identified. We then analyzed these mutants in the next generation. One mutant 23-3 showed a reduced sensitivity to CWM treatment at 15 μM and 20 μM (Fig. 3A, B). The 23-3 mutant showed some of the cells detached from hypocotyl surface (Fig. 3C), indicating that the cell adhesion was defective.

Then we conducted map-based cloning using about 200 resistant F2 individuals from a cross between 23-3 (Col-0 background) and Landsberg erecta (Ler-0). The mutation site was mapped to an approximate 170 kb region between the molecular markers 1-AC012680-9666 (1-29.4108 Mb) and 1-AC007260-9724 (1-29.5868 Mb) on chromosome 1 (Fig. 3D). A putative pectin methyltransferase QUA2 was located in this region. We sequenced the QUA2 genomic DNA and found the mutant harbored a CAG to TAG mutation at the 1094th site, leading to an early stop of translation at the 312th amino acid (Fig. 3D). Reintroducing the QUA2 gene into the mutant rescued the chemical genetic and phenotypic changes (Fig. 3A, C). These results further complement the finding that the phenotypes were caused by this QUA2 mutation. Thus we designated the identified mutant 23-3 as qua2-3 following the previous identification of qua2-1 and qua2-2 mutant (Mouille et al., 2007).

3.3. CWM increased pectin content in Arabidopsis hypocotyls and its inhibitory effects could be partially restored by pectinase treatment.

The mutant qua2-3 displayed cell adhesion defects, which was rescued by CWM treatment (Fig. 4A). As qua2 mutation showed a reduction of HG pectin (Krupková et al., 2007; Mouille et al., 2007), we speculated that CWM might affect pectin modification. We employed pollen tubes as a test system to examine the CWM effect on pectin. CWM treatment reduced pollen tube elongation and inhibited pollen germination (Fig. 4B). Branched and swollen pollen tubes were observed after CWM treatment (Supplementary Fig. S4). Pectin staining indicated an increase of esterified pectin after CWM treatment (Supplementary Fig. S4). We also examined other pectin-related gene mutants for their responses to CWM. pmr6-1, a putative pectin-degrading enzyme mutant showed hypersensitive to CWM treatment (Fig. 4D). Total pectin content in hypocotyls was increased two-fold after CWM treatment (Fig. 4E).

Next, we used CWM in combination with pectinase (hydrolysis of pectin) to treat Arabidopsis seedlings, the results showed that without pectinase, dark-grown hypocotyl elongation was inhibited by CWM, and such inhibition was restored with addition of pectinase (Fig. 5A, B). In addition, the squeezed spots on the hypocotyl caused by CWM were eliminated by pectinase treatment in a dose dependent manner. The percentage of hypocotyls with squeezed spots was decreased from 40% to 5% with addition of pectinase (Fig. 5C).

Then we tested whether CWM directly inhibits pectinase activity in vitro. Seed coat mucilage is rich in pectin (Saez-Aguayo et al., 2013). Pectinase effectively eliminated the seed coat mucilage layer and severely inhibited hypocotyl elongation (Supplementary Fig. S5). However, CWM treatment did not inhibit the activity of pectinase on the
seed coat or hypocotyl (Supplementary Fig. S5), suggesting that CWM and pectinase act on pectin via different mechanisms.

3.4. Cellulose synthesis was impeded in the CWM treated hypocotyls

In formation of the cell wall complex, interconnection of pectin with cellulose is critical for cell growth (Wang et al., 2015, 2012; Zykwinska et al., 2008, 2005). Pectin modification may affect cellulose synthesis (Yoneda et al., 2010), though the precise relationship has yet to be elucidated. When hypocotyls were treated with CWM, crystalline cellulose content was reduced substantially (Fig. 6A), suggesting a possibility that CWM inhibited cellulose deposition during cell growth. When cellulose synthase (CESA) mutants ixr1-1, ixr1-2 and ixr2-1 (IXR1/CESA3, IXR2/CESA6) (Desprez et al., 2002, 2001) were treated with CWM, hypocotyl elongation in the ixr mutants was inhibited by CWM. The inhibition was saturated at 5 μM for ixr1-2, a much lower concentration than in the WT treatment (the concentration of IC50 for WT) (Fig. 6B, C). This indicated that the ixr mutants were hypersensitive to CWM.

Next, CWM was compared with the cellulose inhibitor isoxaben for the effect on cell growth. When CWM and isoxaben were used to treat etiolated hypocotyls, both chemicals caused a swollen phenotype on the hypocotyl (Supplementary Fig. S6A-C). Meanwhile, a pectate lyase-like gene mutant pmr6-1, which contains abnormal pectin accumulation (Vogel et al., 2002, 2004), was hypersensitive to isoxaben (Fig. 6D, E), suggesting importance of the precise pectin accumulation for cellulose biosynthesis. However, CWM partly rescued the isoxaben-caused swollen phenotype. Meanwhile, pectinase treatment, which showed a restoration of the CWM inhibitory effect, was unable to restore isoxaben-caused growth inhibition (Supplementary Fig. S6D–F). This suggests that CWM may function in a way different from that of isoxaben. As CWM affected pectin modification, it is possible that the pectin status changes caused by CWM in turn disturbed cellulose biosynthesis during cell growth.

4. Discussion

4.1. CWM interferes pectin modification during cell growth

Precise pectin modifications such as methylesterification and demethylesterification are important for plant cell growth (Foster et al., 2010; Peaucelle et al., 2012). Mutant analyses have provided genetic evidence to understand how pectin modification is related to cell growth. For example, defective pectin methyl-esterification in ga1-3 and gai mutants impedes hypocotyl elongation (Pelletier et al., 2010). Mutation of a PME-homologous protein VGD1 impairs pectin demethylesterification and inhibits pollen tube elongation (Hu et al., 2015, 2005). In this study, we identified a small molecule CWM, which inhibits hypocotyl cell elongation. Genetic screening revealed that qua2-3, which carries a mutation in a putative pectin methyltransferase gene (Krupkova et al., 2007, 2007), has reduced sensitivity to CWM (Figs. 3 and 4). The results suggest that CWM may interact with pectin metabolism.

CWM treatment led to increase of pectin contents while pectinase treatment can partially restore the CWM-caused phenotypes. The genetic evidence showed that CWM may target at the QUA2 related process, while further investigation is needed to elucidate the detail
mechanism how QUA2 function is related to CWM. Study has reported that QUA2 mutant led to reduction of HG pectin but the remaining HG methylesterification was unaltered (Mouille et al., 2007). This suggests that accurate pectin deposition play an important role in cell growth. Although it is proposed that QUA2 functions as a putative methyltransferase that modifies the pectin structure (Mouille et al., 2007), it is still unclear what is the exact nature of QUA2 function in pectin modification. Identification of CWM provides a useful tool to further characterize the function of QUA2 which has repeatedly shown genetic significance in cell growth (Krupkova et al., 2007; Mouille et al., 2007).

4.2. Pectin modification and cellulose biosynthesis

Previous studies suggest that pectin plays a critical role in constructing the polysaccharide network in cell walls (Braybrook et al., 2012; Braybrook and Jonsson, 2016). Pectin is also recognized for its role in regulating cell wall flexibility and cell growth (Hocq et al., 2017; Wang et al., 2018; Wolf and Greiner, 2012). Perturbation of pectin synthesis led to changes of the cell wall structure (Biswal et al., 2018), suggesting that pectin deposition is important for appropriate organization of other cell wall components, such as cellulose.

In this study, CWM treatment resulted in increase of pectin content and decrease of cellulose content in hypocotyls (Fig. 6). In addition, cellulose synthase CESA mutants ixr1 and ixr2 are hypersensitive to CWM (Fig. 6). Possibly, CWM caused disturbance of pectin modification and over-accumulation of pectin, which in turn interferes cellulose biosynthesis. When plant cell elongates, pectin undergoes modification, degradation and structural reorganization, which enable loosening of cell walls and expansion of cells (Haas et al., 2020; Kim and Carpita, 1992). CWM-treatment resulted in the cell wall which is aggregated with more pectin, and the abnormal pectin accumulation may interfere accurate deposition of cellulose microfibrils and cell elongation. In support of this, the pectate lyase–like gene mutant pmr6-1 is hypersensitive to isoxaben (Figs. 4 and 6D, E), suggesting that disruption of pectin accumulation affects cellulose biosynthesis. However, more mechanistic dissection is needed to understand how the CWM-caused pectin disturbance affects cellulose biosynthesis process. Nevertheless, the study provides a line of new evidence in supporting that pectin modification and cellulose synthesis are interconnected.

4.3. CWM may be used as a tool for study of cell wall modification during cell growth

During cell elongation, the interconnection between pectin and other polysaccharides undergoes active modification. A great deal of interest has been attracted to elucidating the mechanisms underlying the structural changes (Cosgrove, 2018; Wolf and Greiner, 2012). To investigate the process of cell wall modification, several challenges have to be overcome: (i) cell growth, cell wall biosynthesis and remodeling are highly dynamic; (ii) the interactions between pectin and other polysaccharides in plant cell walls are remarkably complex (Wang and Hong, 2016); (iii) genes involved in pectin metabolisms are
generally redundant (Senechal et al., 2014). Chemical genetics, using natural or synthesized small molecules to disturb a biological process, can be an effective tool to help us understand the mechanisms of these processes (Schreiber, 2005; Zhang et al., 2009). Many chemicals have been used to explore the mechanisms of cellulose function (Desprez et al., 2002; Lazzaro et al., 2003; Scheible et al., 2001). Finding new small molecules that affect pectin status will accelerate our research on pectin function (Yoneda et al., 2010). Our study identified a new small molecule CWM that causes increase of pectin content and decrease of cellulose content. CWM also displayed properties of being highly sensitive for inhibition of cell growth with stable and easily detectable phenotypes. Thus the tested characteristics and genetic activity make CWM to be a useful chemical tool to investigate the interaction between cell wall polysaccharides and their dynamic modifications during cell growth.

CRediT authorship contribution statement

Wenbo Li: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft. Qian Zhang: Formal analysis, Investigation, Methodology, Validation, Visualization. Lingting Li: Investigation, Methodology, Validation, Visualization. Lili Gu: Investigation, Methodology, Validation, Visualization. Shumin Cao: Investigation, Methodology, Validation, Visualization. Laifu Luo: Conceptualization, Funding acquisition, Resources, Supervision, Investigation, Methodology, Validation. Laigeng Li: Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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