A simple method for exogenously phytohormone application to the protodermal areas in the base of maize (*Zea mays* L.) leaf

Jieping Li (ljp905@126.com)
Henan University https://orcid.org/0000-0002-3514-5467
Xinlei Feng
Henan University

Methodology

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Abstract

Background: The maize leaf epidermis is function as protection against water loss and gas exchange, contacting the environment and avoiding the damage, which is an attractive system for studying the process of cell fate and development. In monocots, leaves epidermis grown from basal meristem, which contains protodermal cells. The leaf protoderm zone was covered by the leaf sheath or coleoptile in maize, the classic exogenously phytohormone application method, such as spraying on leaf surface or adding in the culture media can't apply the phytohormone to the protoderm areas directly, which restricts the research about phytohormone effect epidermal development.

Results: Here we described a simple and direct method for exogenously application of phytohormone to maize leaf protoderm. We use the auxin analogs 2,4-D to test the system, and the asymmetrical division events which initial stomata development were decreased and the subsidiary cells were induced in advance after 2,4-D treatment. This result was the same as other similar studies' results, indicated that the method is suitable for been used for application phytohormone to the maize leaf protodermal areas.

Conclusions: The method, applied hormones on the mesocotyls of the maize seedlings, is simple and direct. Only a small amount of externally applied substances is required to complete this experiment through this method. The entire experiment process just last 10 days generally and it is easy to survey the phytohormone's effect on the epidermis development**.**

Background

This leaf epidermis is outer layer of leaves, which has multiple functions: preventing water loss[1], regulating gas exchange and secreting metabolic compounds. For maize, it includes several differentiated cell types: epidermal cells, guard cells, subsidiary cells and trichomes. The stomata are small pores, which scattered on the upper and lower epidermis. It is made by two dumbbell-shaped guard cells flanked by two subsidiary cells in maize[2, 3]. The stomata play a role in photosynthetic gas exchange and transpiration[4, 5, 6]. Many phytohormones had been proved to be involved in the development of stomata in dicots. For example, auxin negatively regulates stomatal development[7, 8, 9, 10, 11]. The decreased levels of auxin in the smaller daughter cell improves the equal division of the guard mother cell to produce the two guard cells. Cytokinin increased numbers of stomata per leaf area in tomato and brassinosteroid inhibited stomatal development[12, 13, 14, 15].

The grasses are key species for food, fuel, and the global environment. It is important to reveal the mechanism of grasses leaf epidermis development. The knowledge of the effects of phytohormones on the development of grasses leaf epidermis is limited. For maize, added indole-3-acetic acid (IAA) and 1-napthaleneacetic acid (NAA) exogenously promoted the establishment of subsidiary mother cell (SMC) polarity and subsidiary cell formation[16]. But the molecular mechanism of the phytohormone's effect on development of monocot leaf epidermis is very few. One reason is the development and distribution of leaf epidermal cells in monocots and dicots are very different. In dicots, stem cell-like stomatal precursors

are scattered in the leaf and distributed throughout the leaves, which divided throughout the leaf and promote the typical “broadleaf” or radial growth characteristic of these plants[17]. In monocots, the basal meristem region is located at leaf base, there are two zones above the meristem, the zone of stomatal initial origin and the zone of stomatal differentiation[18]. The cells in below basal meristem intercalary growth make the up cells move out of the meristematic region. Epidermal cells pass through different stages of development and maturation. Maize leaf epidermal cells are typically rectangular and occur in files parallel to the leaf venation, just like rice[19, 20]. The method spraying exogenously phytohormones on leaf surface, which was widely used in dicots to study the phytohormones’ effect on epidermis cells development, was not suitable for monocots.

The protoderm in maize is under leaf sheath, there is no simple method to apply phytohormone exogenously[21]. The traditional methods were always addition the phytohormone into the culture media, such as MS medium or nutrient solution, or spraying on leaves, which operating procedure is very complicated and not directly enough[22, 23]. This study described a detailed method for applied the phytohormone to the maize seedling to survey the effect of the hormone’s influence on development of maize leaf epidermis. The total experiment process just last 10 days generally and the method is simple and easy to survey the phytohormone‘s effect on the epidermis development. Auxin analogue 2,4-D was used to test this system, and exogenous application of 2,4-D repressed the cell division in leaf meristem zone and decrease guard mother cells number.

Materials And Methods

Maize seedlings germination

Maize (*Zea mays* L.) inbred line B73 or W22 kernels which were full and mildew-free were selected for planting in a sterile mixed culture matrix (vermiculite: nutrient soil: black soil, 1:1:1). The planting depth is 2cm from the soil surface. 4 kernels are planted in a square box of 5×5×5 cm^3, 12 boxes are placed in each 20×35cm^2 plate, a total of 48 seeds were planted in one plate (Fig. 1A). 100ml sterile water is added at the day of planting to keep the whole soil medium moist. The plate was cultured in incubator with follow parameter: temperature 26/23°C, photoperiod 16/8 h light/dark cycle. Three days after planting, 100ml sterile water was added at the second time. After five days, the coleoptile of maize seedlings can be seen stretching out of the soil surface. One week after sowing, the first leaf had just exposed the coleoptile (Fig. 1A).

Methods for exogenously application of phytohormone

At 7 days after sowing, the coleoptiles are approx. 2 cm long, the first leaf is grown out of the coleoptiles, uniform seedlings are selected for phytohormone treatment. Take the whole seedlings out of the soil, remove the floating soil on the surface, and rain the soil with water. Use scissors to cut off the primary roots and seminal roots (Fig. 1B). And keeping the seedlings in water-containing absorbent paper to prevent dehydration of the young tissues. Wrap the absorbent cotton containing externally applied hormones on the mesocotyls of the maize seedlings (Fig. 1C), and keep a part of the absorbent cotton

sticking out, add 400μl treatment solvent to the self-made 2.5ml centrifuge tube (Fig. 1D), and plant the seedlings containing absorbent cotton in the centrifuge tube to keep the stretched absorbent cotton is in contact with the treatment solvent to divert and externally apply phytohormones for continuous treatment. Use a parafilm to seal the centrifuge tube to avoid volatilization (Fig. 1E), and place the processing device in the incubator (temperature 26/23°C, photoperiod 16/8 h light/dark cycle) to continue the culture. Additional control experiments were performed, control seedlings were placed on cotton wetted with the solvent (distilled water or dimethyl sulfoxide, and the maximum dimethyl sulfoxide concentration solutions was below 0.5% (v/v)). Experiments were repeated at least three times.

Anatomy and phenotype investigation of maize leaf

After treated for 48 hours, the maize seedlings were taken out of the processing device (Fig. 2B), and the stems of the seedlings were dissected using forceps and scalpel blades, and the coleoptile and first leaf, which were wrapped in the outer layer, were removed (Fig. 2D). Carry out a detailed dissection of the second leaf, save the sample which contain the parts of 1cm from the base of the stem, place the sample in 10 mg/ml propidium iodide (PI, Beijing Solarbio Science & Technology Co., Ltd) staining dye solution for 5 minutes (Fig. 2E). After staining, pick out the sample, remove the dye attached to the surface of the sample with water, and place it on the glass chip. The phenotype was observed under a confocal microscope (Fig. 2F). PI is applied to stain cell walls, which fluorescence excitation maximum is 535 nm and the emission maximum is 617nm[24].

A series of overlapping images was taken along the leaf apex by a fluorescence confocal microscope (Nikon A1, Nikon, Japan). The length and width of different type cells were measured using ImageJ software.

Results

Stomatal development events in maize

The development of maize stomatal complexes followed sequence events, which occurrence in the leaf basal meristem region. The series events begin with cell proliferation of meristematic cells which were found in the basal meristem region beside preligule band (PLB) (Fig. 3A)[25]. The remaining series events happened in the zone of stomata initial origin and the zone of stomatal differentiation, which are two consecutive zones above the meristem (Fig. 3A).

The development of stomatal in maize can be divided into six consecutive stages (Fig. 3B2-3B7)[26, 27]. It contained three asymmetrical divisions and one symmetrical division process. Initially, potential precursor cells proliferate in particular files at the distal end of the meristematic cell close to leaf base (Stage 1, Fig. 3B2). A stomatal initial from the first asymmetrical division produce the guard mother cell (GMC), which separated a small rectangular cell toward the leaf tip (Stage 2, Fig. 3B3). Subsequently, a pair of subsidiary cells were induced by the GMC asymmetrical divisions in the laterally adjacent subsidiary mother cells (SMC) (Stage 3 and 4, Fig. 3B4 and 3B5), finally the GMC then symmetrical

division into two immature guard cells(GC) (Stage 5, Fig. 3B6). Four especially cells mature and expand to form a mature stomatal complex (Stage 6, Fig. 3B7).

Leaf tissue was extract from 10 days old maize seedlings, its leaf 2 was just emerging from the whorl (Fig. 2D). The coleoptille and leaf 1 were removed to expose developing tissue at the bases of leaf 2; There are three zones at basal leaf zones were excised for analysis based on the developmental stages they represented (Fig. 3A). Zone 1 is located at the base of about 80μm, it contains preligular band(PLB) structure, which are dividing cells with shape of primarily isodiametric. Zone 2 is between 100μm to 5500μm from the PLB, contains differentiation cells, which are varying size and shape cells. It contains the pathway of stomata development. The length of total cell at asymmetric entry division stage is about 350μm, and the SMC formation stage is about 400μm, the SMC asymmetric division is about 1600μm and the GMC symmetric division stage is 2700μm. Zone 3 is about 5500 μm away from the ligule, contains extensive differentiation cells which expand to mature stomata (Supplementary Fig. 1).

Exogenously application of 2,4-D (0.4mg/ml) repress the cell division in leaf meristem zone, decrease number of GMCs and make ligule disappear at leaf base.

The application of 2,4-D in this work influence the growth of maize seedling leaves. It mainly make the ligule disappear and a remarkable decrease in GMC formation. In the protodermal areas at leaf base, GMC number and the number of epidermal cells were decreased and the length and width of epidermal cell was increase comparable to the mock treatment at the asymmetric enter division stage. Some young subsidiary cells were induced by GMC, which is more closed to the leaf base than the control leaves (Fig. 4B3). Collectively, these data indicated that 2,4-D suppressed the development of ligule and decreased the number of cell entry into the first asymmetric division and suppressed the cell symmetric division.

There is a uniquely linear band, located at leave base, which will develop to ligule and auricle, named preligule band (PLB). The cells in PLB are smaller and run perpendicular to the proximal-distal axis of the developing leaf (Fig. 3B1 and 4A1). Cells periclinal divisions in PLB will develop to ligule, which are parallel to the leaf surface[28, 29]. On the developing ligule stage, the PLB was disappeared at 48 hours after 2,4-D treatment. And the smaller cells in PLB zone, which was characteristic of PLB cells were reduced. The cells number in this zone were also decreased significantly (Fig. 4B1).

At the cell proliferation stage, the cells number per area was less than mock treatment group (Fig. 4B2). And the width and length of cells in this zone was larger than mock treatment group. The average length of cells is 26.77μm after 0.4mg/ml 2,4-D treatment, is longer than mock significantly, which value was 17.47μm (Fig. 5A). And the average width of cells in 2,4-D treatment group (13.91μm) is also significantly larger than mock(12.19μm) (Fig. 5B).

At asymmetric entry division stage, the number of GMCs in 2,4-D treatment group was less than mock, and the symmetric division was also reduced compare to mock. The number of guard mother cells (GMC) was reduced. The density of GMCs was 5.6 per 160 ×160μm^2 area leaf after 0.4mg/ml 2,4-D treatment

and it was 16.6 at the mock treatment (Fig. 4B3, Fig. 5C). This result indicated that the first asymmetric division for GMCs was suppressed by 2,4-D. The events of periclinal division and perpendicular division were also reduced, it means that the division event of asymmetric and symmetric were suppressed by 2,4-D. The cells number on 2,4-D treatment group was less than mock group, it may be induced by decreased cell division. This may lead the length and width larger than mock and the cell number was less than mock. Additionally, there are some GMCs induce the SMCs at this stage, the young stomata complex formation more closed to the leaf base.

At SMC formation stage, there are some GMCs induced the two SMCs, and produce unnormal stomata complex which include two young subsidiary cells(YSCs) in 2,4-D treatment group (Fig. 4B4). And the stomata complex in mock group is include mature GMC and flanked by two nascent SCs (Fig. 4A4).

At SMC asymmetric division stage, the number of cells and stomata in 2,4-D treatment group was similar as mock treatment group (Fig. 4B5). It indicated that the 2,4-D did not influence the development of stomata on this stage, maybe because the initiate process of stomata at this stage had finished before 2,4-D treatment. It indicated that initial process of stomata may be influenced by 2,4-D, but when the initial process had been finished, the GMCs will develop into the mature stomata complex even under the condition of 2,4-D treatment.

The minimum concentration of 2,4-D which influence the formation of subsidiary cell in maize is $4x10^{-3}$ mg/ml.

At the asymmetric entry division stage, there are many young subsidiary cells were induced by GMCs in the 2,4-D treatment group at a concentration of $4x10^{-3}$ mg/ml (Fig. 6C2). And there are many GMCs induced the subsidiary cells at $4x10^{-2}$ mg/ml and $4x10^{-1}$ mg/ml (Fig. 6D2, Fig. 6E2). The young GMCs at the asymmetric entry division stage didn't induce the subsidiary cells under 2,4-D treatment conditions at a concentration of $4x10^{-4}$ mg/ml (Fig. 6B2), just like the young GMCs in mock treatment group. This result indicated the minimum treatment concentration that affects formation of subsidiary cell is $4x10^{-3}$ mg/ml for 2,4-D treatment.

At cell proliferation stage, there are many GMCs which are produced by asymmetric division under 2,4-D treatment conditions at a concentration of 0.4mg/ml (Fig. 6E1). For other concentration from $4x10^{-2}$mg/ml to $4x10^{-4}$mg/ml, the cells in stomatal lineage proliferated just like the cells in stomatal lineage in the mock treatment group (Fig. 6B1-6D1), it indicated that when the 2,4-D concentration is higher than 0.4mg/ml, the asymmetric division is closer to the leaf base.

Discussion

In maize, the growth process of leaves is a linear organization, dividing cells at the base zone of the leaf, followed by expanding cells and finally maturation cells at the tip[30]. For a layer cells, epidermis can be treated as one-dimension. Cells in maize leaf epidermis occur division and expansion along the axis

jointly. Cell division process provide more new cells. After cells have ceased to divide, cell expansion process, greatly increases the cellular volume[30, 31]. The base zone with dividing cells in leaf epidermis always named protoderm[32]. Leaf protoderm play a key role in the development of leaf epidermis. And it represent ideal model systems for quantitative studies for the spatial patterns of cell division and cell expansion, which is useful and simple research system in plant developmental biology.

Phytohormones, and small molecular substances with biological activity, such as small molecular peptides, regulate the development of plants and participate in the development of leaf protoderm. Application of these regulatory substances is an effective method to study the molecular mechanism of protoderm development. But the protoderm of monocot plants is covered by leaf sheaths, the traditional method of spraying on the surface of leaves can affect the development of leaf, such as GA is sprayed on the leaf surface of dwarf mutants can rescue mutants phenotype[33]. Spraying or immersing the synthetic 6-benzylaminopurine (BA) OR ABA to tomato, is suitable to dicots, which meristemoids are cover the full leaf[34]. But the spraying operation is not targeted, and it is generally used for long-term phenotypic observation, the quantification and observation time of the externally applied substances are not clear, and a large amount of externally applied substances is required, which is not suitable for some precious reagents. Another used widely method for exogenously phytohormone application is addition phytohormones into the culture medium. Many phytohormones had used this method to application in the plant, such as brassinosteroid[35] and auxin[7]. The root system absorbed phytohormone into the plant body, and the root system is affected first. The unnormal roots will affect the absorption of nutrients and water, and it may affect the normal growth of the plant, cause abnormal development of the protoderm, which is not a direct result of the externally applied substances. Moreover, aseptic operation always be required, the process is complicated, and the experiment cycle is long.

This method reported in this paper, phytohormone was applied externally to the maize mesocotyls, and the root system was removed to avoid its physiological effects, and the adsorption effect of absorbent cotton was used to maintain the quantitative concentration of phytohormone. It can be processed with very little reagents. The treatment time of external application is always 2 days. During the treatment, the externally applied hormone maintains a stable concentration, the treatment operation is simple, and the phenotypic observation method is quick and convenient, which is suitable for the study of the developmental mechanism of maize protoderm.

In this study, exogenous application of 2,4-D repressed the cell division in leaf meristem zone, decrease number of GMCs and make ligule disappear in maize seedlings. Auxin is one of master plant developmental hormones. it controls stem cell compartment size in stomatal development in *Arabidopsis*. The PIN-FORMED protein mediated auxin polar cell-to-cell transport, PIN-GFP fluorescence is especially strong around stomatal precursor cells, meristemoids and guard mother cells. High efflux of auxin from meristemoids, mediated by PIN3, is required for fate transition from the meristemoids to guard mother cells. Some PINs mutants show a defect stomata pattern. Cell fate transit in stomata development process probably required PIN-mediated transport of auxin. Auxin and the auxin-responsive gene Monopteros(MP) work together to repress the express of STOMAGEN in mesophyll. The

STOMAGEN work as mobile peptide improve the development of stomata. 2,4-D treatment decrease STOMAGEN expression significantly in *Arabidopsis* at seedling stage. MP binds to the stomagen promoter directly and the genetical studied about STOMAGEN-RNAi; *arf5-1*/+ transgenic line indicated that MP regulated stomata development through repressing STOMAGEN expression[36]. In this study, 2,4-D repressed the asymmetric division at asymmetric entry division stage in maize, whether the molecular mechanism behind this physiological phenomenon is similar to *Arabidopsis* required further research works in future.

Auxin was a key player in generation of subsidiary cells in maize. Spatiotemporal changes of PIN protein indicated local auxin transfer from GMCs to SMCs[16]. Combined with our experimental results, the auxin play key role in SMC establish polarity. In maize, *ZmMUTE*, a bHLH transcription factor, plays a vital role in determining the fate of SMC. It induced SMC polarization and regulated the differentiation of the last symmetrical division of GMCs, thereby producing the two GCs. It is an important regulator of PAN1 and PAN2. PAN2 is polarized in premitotic SMCs[37]. PAN1 and its downstream gene ROP2/9 proteins are polarized after PAN2 polarization. All of them work together with F-actin to induce nuclear migration towards the GMC proximal site and make SMC polarity[37, 38, 39]. In this study, there are many young subsidiary cells were induced by GMC even at the asymmetric entry division stage of stomata development in the 2,4-D treatment group at a concentration of $4x10^{-3}$ mg/ml, it indicated that the 2,4-D improved the formation of SMC. More works could be carried out to clarify the relationship between the 2,4-D and *ZmMUTE*, *PAN1*, *PAN2*. The express level of those after 2,4-D treatment would be observed, and the phenotype of mutants of *pan1*, *pan2* and *ZmMUTE/BZU2* would be surveyed after 2,4-D treatment to uncover the mechanism of 2,4-D improve the SMCs induced.

Conclusion

This paper presented a simple and convenience method for exogenously application of phytohormone to the protodermal areas in the base of maize (*Zea mays* L.) leaf. Put each 5 days after germination seedling separately in a self-made processing equipment, and use absorbent cotton to maintain a continuous supply of external reagents for 3 days. After treatment, the cell wall was staining by PI, and the phenotype was investigated by Confocal system. The entire experiment process just last 10 days generally and it is easy to survey the phytohormone's effect on the epidermis development. The auxin analogs 2,4-D was used to test the system, and it repressed the asymmetrical division events which initial stomata development and induced the subsidiary cells. Exogenously application of 2,4-D (0.4mg/ml) repressed the leaf meristem cell division, decreased number of GMCs and made ligule bland disappeared. This result was the same as previous similar studies, indicated that the method is suitable for application phytohormone to the maize leaf protodermal areas.

List Of Abbreviations

IAA, indole-3-acetic acid

NAA, 1-napthaleneacetic acid

SMC, subsidiary mother cell

PI, propidium iodide

GMC, guard mother cell

GC, guard cells

PLB, preligular band

YSC, young subsidiary cell

BA, 6-benzylaminopurine

MP, Monopteros

Declarations

Authors' contributions

Jieping Li: Formulate the idea, designed the project, carried out most of the data analysis, draft the manuscript. Xinlei Feng: carried out the microscopy experiments and contributed to the data analysis. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Maize seedlings germination and application of phytohormone exogenously A. Maize seedlings germinate in the square culture box. B. Maize seedlings with main roots and embryo roots were cut off. C. Absorbent cotton contain externally hormones on the mesocotyls of the maize seedling. D. Self-made 2.5ml tubes. E. Treatment device.

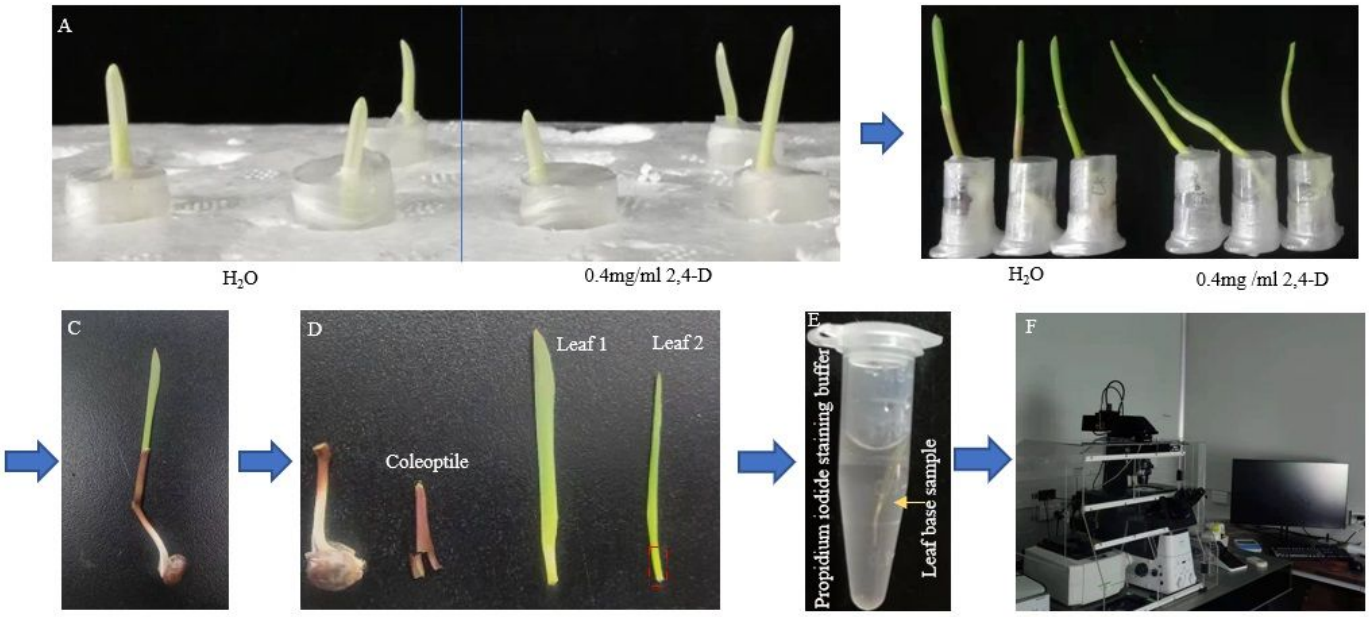

Figure 2

Anatomy seedlings and phenotype investigation A: Maize seedlings treated with 0.4mg/ml 2,4-D. B: Maize seedlings treated with 0.4mg/ml 2,4-D after 36 hours. C: Maize seedling after treatment. D: Anatomy the seedings and collect the leaf base samples (in red dotted frame). E:The leaf base samples were stained by propidium iodide. F: Investigate the phenotype under the confocal microscope.

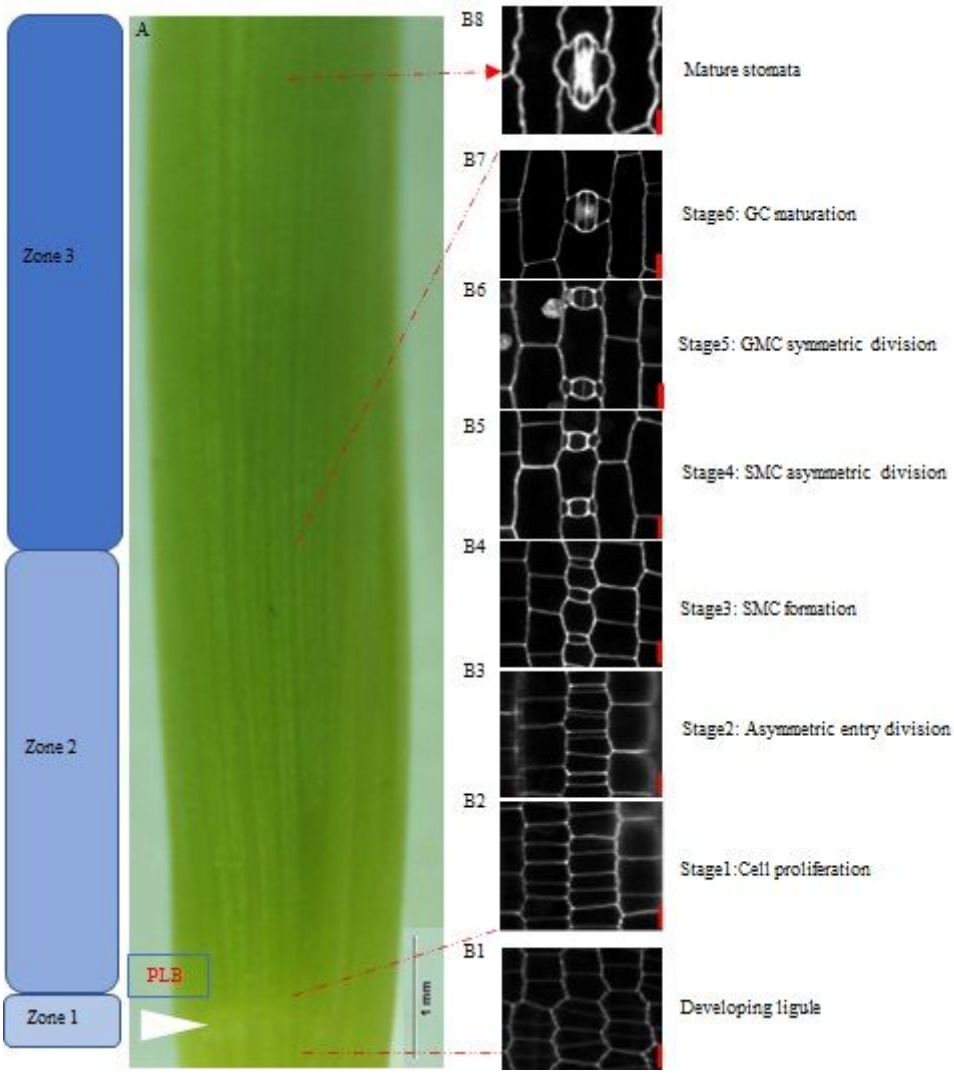

Figure 3

The development process of stomata in maize A. Zone 1 at the leaf base contains the developing ligule. Zone 2 contains the sequence series events of stomata development. Zone 3 is mature leaves which are fully expanded with mature stomata (B8). The white arrow indicated the preligule band (PLB, B1). Cell proliferation for stomatal lineage cells (B2). Small guard mother cells (GMCs) were generated by asymmetric division (B3). Expansion GMC induce the subsidiary mother cells (SMCs) formation (B4). SMCs formation via asymmetric divisions and maturation (B5). GMCs divide symmetrically product immature guard cells(GCs) (B6). Young guard cell expansion and elongation to form guard cell complex(B7, B8). Confocal images of propidium iodide stained were taken from the base of leaf 2 of 6 days after growing seedling of maize (B73). Red scale bar=10μm.

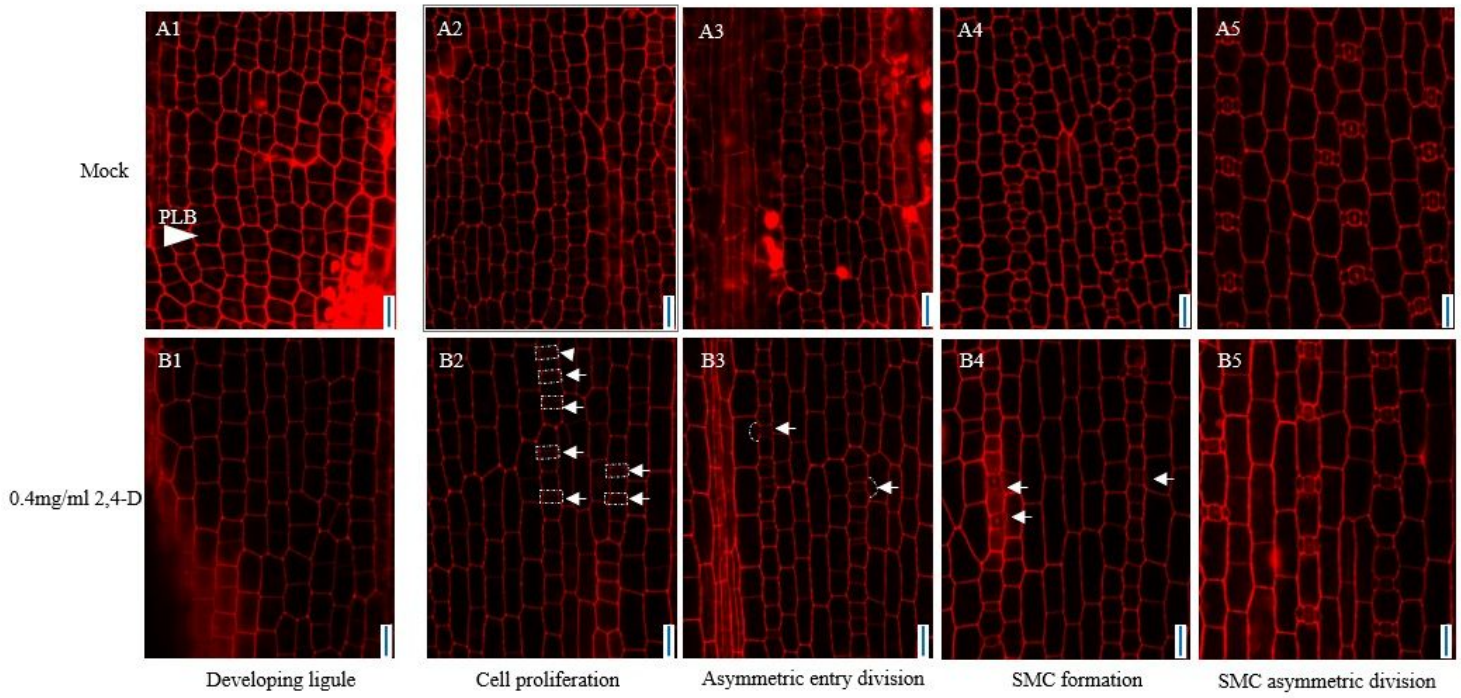

Figure 4

Confocal images of leaf protoderm treated with 2,4-D (0.4mg/ml) and mock A1-A5 Mock treatment group; B1-B5 0.4mg/ml treatment group, The ligule was shown in A1 with white arrow. Ligule was disappeared in B1 at the leaf base. The number of GMC at Asymmetric entry division stage (B2) were decreased. And the number of young stomata at SMC asymmetric division stage (B3) were decreased. Unnormal stomata complexes formed at the SMC formation stage in 0.4mg/ml treatment group (B4). All confocal images were stained by propidium iodide. Scale bar= 20μm.

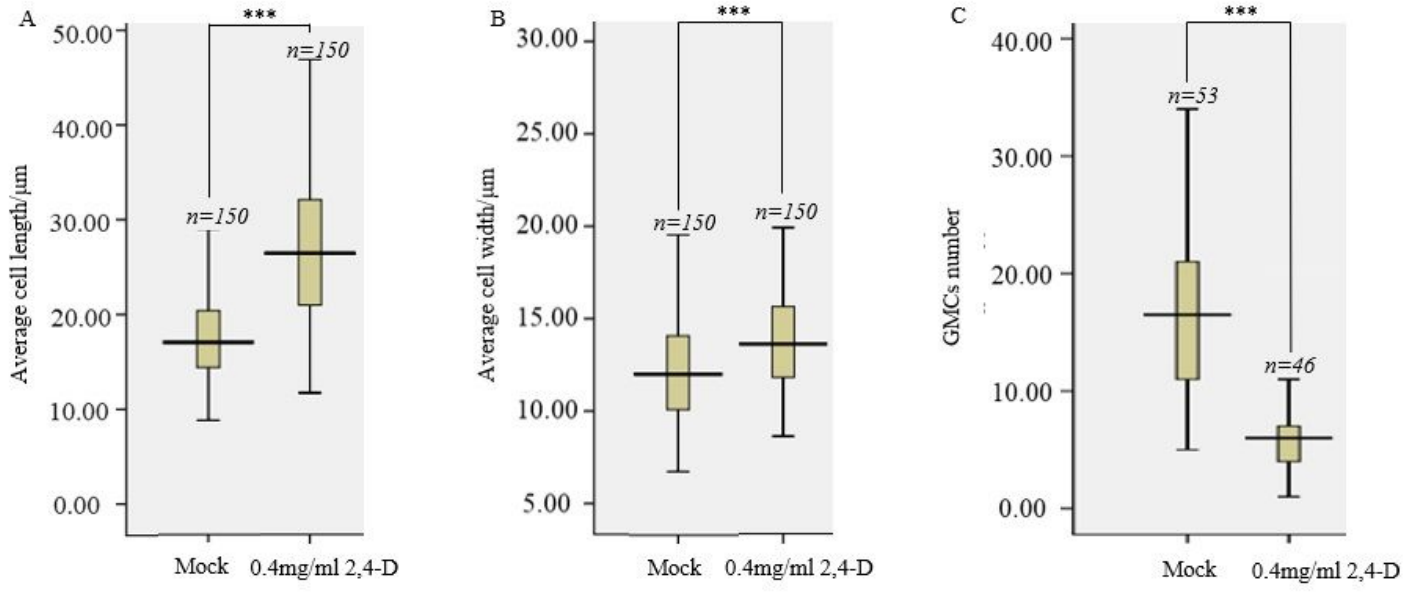

Figure 5

Characteristics of the cells at protodermal areas after 2,4-D treatment A: The average cell length at cell proliferation stage after 2,4-D (0.4mg/ml) treatment was larger than mock significantly. Bars indicate mean (±SD) (n =150). B: The average cell width at cell proliferation stage after 2,4-D (0.4mg/ml) treatment was larger than mock significantly. Bars indicate mean (±SD) (n = 150). Significant difference within each group was indicated by an asterisk (***p < 0.001). C: The GMCs number was repressed after 2,4-D (0.4mg/ml) treatment. (***p < 0.001) according to Student's t-tests.

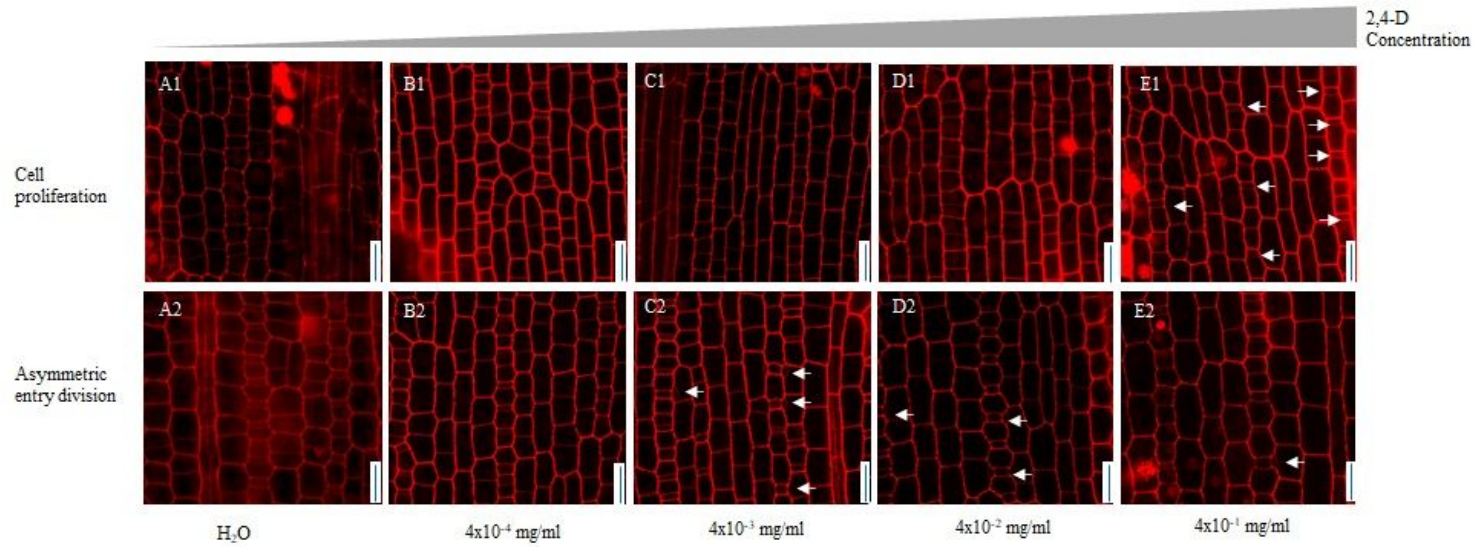

Figure 6

Confocal images of leaf protoderm treated with different concentration of 2,4-D (10-4~10-1 mg/ml) A1-E1 the cell proliferation stage of leaf protoderm area of treatment with different concentration of 2,4-D (10-4~10-1 mg/ml). A2-E2 the asymmetric entry division stage of leaf protoderm area of treatment with different concentration of 2,4-D (10-4~10-1 mg/ml). All confocal images were stained by propidium iodide. Scale bar= 20μm.

Supplementary Files

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- SupplementaryFigure.pptx