Morphological Study on the Incidence of Intumescence Injury in Tomato Plant Leaves

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Intumescence injury of tomato leaves is a physiological disorder in which white surface spots rise on the abaxial side of the leaf and then turn brown; when it worsens, the leaf dies. Scanning electron microscope observations showed individual cells were enlarged and raised due to intumescence injury. Vein epidermal cells and stomatal guard cells were also enlarged. Hypertrophy was observed by light microscopy in epidermal cells, spongy tissue cells, and palisade tissue cells. In thin leaf sections prepared with Technovit 7100 resin, the resin peeled from the epidermal surface on the adaxial and abaxial sides of normal leaf areas. However, resin did not peel off from the enlarged epidermis in areas of intumescence. Transmission electron microscopy imaging of the cuticular layer in the epidermal cells affected by intumescence injury showed disappearance of the outermost wax layer, abnormalities in intermediate layer, and decrease in cuticular layer. Toluidine blue O staining was used to identify abnormalities in the cuticular layer and the area of intumescence, which was stained purple. The percentage of staining area relative to total area of the leaf was used to quantify the degree of intumescence. In experiments using incubators under low-UV conditions, intumescence injury was less likely to occur in young leaves or fully expanded leaves and more likely to occur in expanding leaves. In addition, the water content of the leaves in which intumescence injury occurred was lower than that in normal leaves. Intumescence injury occurred less frequently under conditions of 70% relative humidity than under conditions of 90% relative humidity. UV and relative humidity are known to affect the development of the cuticular layer, and intumescence injury was severe under conditions in which the cuticular layer had difficulty developing. Our findings showed that intumescence injury of leaves was accompanied by abnormal cuticular layer, and suggest that occurrence of intumescence injury may be reduced under conditions in which the cuticular layer is well developed, such as with UV irradiation and low relative humidity.

Key Words: anatomy, cuticular layer, electron microscope, relative humidity, ultra violet.

Introduction

In Japan, ultraviolet (UV)-cut films are widely used to cover greenhouses for the purpose of pest control in tomato cultures (Sasaki and Nakanura, 2005; Futamura and Kumazaki, 2012). When UV-cut film is used in tomato cultivation, intumescence injury, which rises above the tissue on the leaf surface like a tumor, is sometimes observed and it can lead to reduced yield of tomato plants.

Intumescence injury, also known as edema, is a physiological disorder characterized by abnormal outgrowths of epidermal and/or palisade parenchyma cells on the leaf, petiole, or stem surfaces of the affected plants. Intumescence injury is a common problem when growing a wide variety of plants under artificial light (Williams et al., 2016); and was reported to occur in UV-deficient environments, such as those in which light-emitting diode (LED) sole-source lighting supplies only red wavelengths (Heidi and Runkle, 2015). High relative humidity can cause intumescence injury in some plants. For example, the disorder was induced in Eucalyptus species by high relative humidity (RH), but was not present in plants grown continuously at RH < 60%–65% (Warrington, 1980). However, Lang and Tibbitts (1983) found severe symptoms of intumescence injury in tomato plants regardless of RH (30% vs. 92%). Therefore, in the present study, we investigated the occurrence of intumescence injury under differing conditions.
relative humidity conditions.

Morphological studies on intumescence injury were conducted on tomato plants (Lang et al., 1983; Zhao et al., 2008; Craver et al., 2014), *Eucalyptus* (Pinkard et al., 2006), ivy geraniums (Rangarajan and Tibbitts, 1994), ornamental sweet potato plants, interspecific geraniums, and bat-faced cuphea (Craver et al., 2014). In tomato plants, intumescence injury consisted primarily of the following symptoms: gall-like tissue on the petioles, midribs, and stems; hypertrophy of the epidermis and underlying 2–3 cell layers; and intumescences (Lang et al., 1983). Susceptibility to intumescence injury varies among cultivars; the susceptible tomato cultivar exhibited hypertrophy of the lower epidermis, spongy parenchyma, upper epidermis, and palisade cells (Zhao et al., 2008). In *Eucalyptus globules*, necrosis and cellular collapse of the palisade mesophyll and deposition of phenolic compounds were observed in intumescence areas (Pinkard et al., 2006). The morphological characteristics of edema and intumescence injury may be similar or differ, depending on the species; for example, lesions on geranium and tomato plants occur predominantly on the abaxial surface of the leaf, whereas lesions on ornamental sweet potato and bat-faced cuphea plants occur predominantly on the adaxial surface (Craver et al., 2014). Although some morphological studies exist (as described above), no study yet focused in detail on the surface of the intumescence area or made observations using a transmission electron microscope (TEM). Using TEM, the structural changes in the cuticular layer on the surface of expanding cells, which are likely to cause intumescence injury, may be determined.

In the present study, we examined the epidermal cells of the raised parts on tomato plant leaves using a light microscope, a scanning electron microscope (SEM), and a TEM. For the first time, we report the use of a TEM to focus on the cuticular layer of tomato leaf surfaces with intumescence, and we also report abnormalities. The plant cuticle is an extracellular hydrophobic layer that covers the aerial epidermis of plants, providing protection against external environmental stresses, including desiccation, temperature, new terrestrial extremes, gravity, and increased exposure to UV radiation (Yeats and Rose, 2013). Here, we discuss the relationship between intumescence injury and the cuticular layer in tomato plants. In addition, we show the degree of intumescence may be quantified as a numerical value using the area of the abnormal cuticular layer stained by toluidine blue O aqueous solution.

Materials and Methods

**Experiment 1: Anatomical study of intumescence**

1.1. Light microscopic observation of intumescence

*Solanum lycopersicum* tomato seeds (‘CF Rinka 409’; Sakata Seed Corporation, Yokohama, Japan) were sown in seed trays on September 21, 2011, and grown in a nursery system (Nae-Terrace; Mitsubishi Chemical Agri Dream Co., Ltd., Tokyo, Japan). The seedlings were transplanted into a rockwool cube (75 × 75 × 75 mm; Grodan Delta, Grodan BV, Roermond, the Netherlands) with a nutrient solution (Ohtsuka Ekihi SA formula 1/2; OAT Agrio Co., Ltd., Tokyo, Japan). In an experimental greenhouse, “NARO Tsukuba Factory Farm” (Higashide et al., 2014) covered with a fluoropolymer film (F-CLEAN Diffused; AGC Inc., Tokyo, Japan), 16 plants were transferred to a space with UV-cut film (Easter UV Cut; Mitsubishi Chemical Agri Dream). The wavelength of transmitted light measured using UV-Visible/NIR spectrophotometer (v 600; JASCO Corporation, Hachioji, Japan) is presented in Supplementary Figure S1. Leaf fragments including the intumescence area were fixed in a mixture of 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 20 h at room temperature. After being washed with rinsing buffer (0.2 M phosphate buffer, pH 7.2), the leaf fragments were then dehydrated in a graded alcohol series and embedded in Technovit 7100 resin (Heraeus Kulzer GmbH, Wehrheim, Germany), whose main component is glycol methacrylate. Leaf fragments were cut into semi-ultrathin sections (2 μm) using glass knives on an ultramicrotome (EM US7; Leica Microsystems Ltd., Wetzlar, Germany), and the sections were stained with toluidine blue O. For histological examination, the sections were observed under a BX50 optical microscope (Olympus Corporation, Tokyo, Japan).

1.2. TEM observation of intumescence

The ‘Rinka 409’ tomato seeds were sown in seed trays on June 20, 2014, and grown in a nursery system for 3 weeks. The seedlings were transplanted into a rockwool cube in the greenhouse at Shizuoka University, Japan. Nutrient solution (EC 1.5 dS·m⁻¹, Otsuka SA formulation) was irrigated over time. Five plants were covered with UV-cut film (Disaster UV Cut; Mitsubishi Chemical Agri Dream) for 1 week in the same greenhouse. The wavelength below 380 nm of light inside UV-cut film was little detected by a miniature spectrometer (USB4000; Ocean Optics Inc., Largo, FL, USA). The leaf fragments that included the intumescence area were fixed in a mixture of 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 6 h at room temperature. After washing with rinsing buffer (0.2 M phosphate buffer, pH 7.2), the samples were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h at 4°C. They were then dehydrated in a graded alcohol series and embedded in epoxy resin. For the TEM evaluation, ultrathin sections were prepared using diamond knives on the microtome. The sections on grids were stained with Ti Blue (Nissshin EM Co., Ltd., Tokyo, Japan) for 20 min, followed by a lead electron staining solution (Katayama Chemical Industries Co., Ltd., Osaka, Japan) for 5 min. The sections were observed using a
JEM-1400 Plus electron microscope (JEOL Ltd., Akishima, Japan).

1.3. SEM observation of intumescence

The tomato leaf fragments processed in Experiment 2 (detailed below) that exhibited symptoms of intumescence injury were observed using SEM. On July 10 and 25, 2017, the abaxial surface of raw leaves was observed in a low-vacuum mode using a SEM (JSM-6510LV; JEOL).

Experiment 2: Investigation of the leaf stage and conditions associated with intumescence

2.1. Effects of low-UV duration on intumescence at different leaf positions and plant developmental stages

‘Rinka 409’ tomato seeds were sown on a rockwool multiblock in an incubator (day/night: 30°C/25°C, 12 h light/12 h dark, RH = 80%). Three days later, they were transferred to a glass greenhouse under natural UV conditions at Shizuoka University, Japan, and supplied from the bottom with a nutrient solution (EC 1.35 dS·m$^{-1}$). Subsequently, the plants were placed in an incubator at 28°C, 12 h light/12 h dark, and 85% RH. Fluorescent lamp (HFB32EX-N-H; Panasonic Corporation, Kadoma, Japan) was used. The photosynthetic photon flux density (PPFD) near the plants in the incubator was measured by a quantum meter (IKS-102; Koito Manufacturing Co., Ltd, Tokyo, Japan) was about 200 μmol·m$^{-2}$·s$^{-1}$. Light wavelengths below 400 nm in the incubator were barely detected by a miniature spectrometer.

Tomato plants were sown on May 8, 2017, and transferred to the glass greenhouse on May 11, where they were placed in an incubator 7, 14, or 21 days after sowing for 1 to 4 days. We varied the time at which the plants were placed in an incubator under low UV conditions to investigate whether various leaf stages were sensitive to intumescence.

Following morphological examination, we observed that the cuticular layer was affected at sites where intumescence injury occurred; therefore, we used a simple staining method to stain the undeveloped portion of the cuticular layer (Tanaka et al., 2004). Tomato leaves were immersed in 0.1% toluidine blue O aqueous solution for 1 min and then gently washed in running water. The stained leaf was spread on a transparent acrylic plate, and a digital image was made using a scanner (GT-F670; Seiko Epson Corporation, Suwa, Japan). For each leaf, the degree of intumescence was calculated as the percentage of stained leaf area relative to whole leaf area. The degree of intumescence was calculated by analyzing the digital images using ImageJ software (<http://rsb.info.nih.gov/ij/>). Leaves that died and were swollen due to severe dehydration had a degree of intumescence of 100%.

2.2. Water contents of leaves with intumescence

Tomato plants were sown on June 22, 2017, and transferred to the glass greenhouse on June 25, then placed in the incubator, as mentioned above, on July 11, 2017. In this examination; we examined the degree of intumescence as previously mentioned and water content of each leaf on July 14, 2017. The leaf water content was calculated as follows:

Water content (%) = (Wf − Wd)/Wf × 100

Wf, fresh weight and Wd, dry weight.

The degree of intumescence was calculated as the percentage of stained leaf area relative to whole leaf area as mentioned above.

2.3. Effect of relative humidity on incidence of intumescence

In order to investigate the effects of relative humidity, tomato plants were sown on March 26, 2018, and April 18, 2018, and transferred on March 29 and April 21 respectively to the greenhouse, planted in an incubator set at 28°C, 12 h light/12 h dark, and 90% RH on April 13, or 60%–70% RH on May 7. We managed the plants for 3 days in an incubator at differing relative humidity, and investigated degree of intumescence. The degree of intumescence was calculated as mentioned above.

Statistical analysis was performed using JMP11 (SAS Institute Inc., Cary, NC, USA). Two-way ANOVA was conducted to detect the effect of low-UV duration and leaf positions on the incidence of intumescence at 14 and 21 days after sowing tomato plants, to detect the effect of the UV condition and leaf positions on incidence of intumescence, to detect the effect of the UV condition and leaf positions on the water content of the leaf, and to detect the effect of relative humidity and leaf position on the degree of intumescence, respectively.

Results

Experiment 1: Anatomical study of intumescence

Intumescence injury occurred on leaves of tomato plants covered with UV-cut film. It was caused by abnormally enlarged and raised cells on the abaxial surface of the leaf. By anatomical microscope observation, intumescence was recognized as a white spot on the back of the leaf (Fig. 1A). Later, the raised area expanded, and the surface color of the raised area changed from white to brown (Fig. 1B). As observed by SEM, the normal site was flat, but the epidermal cells with intumescence were raised with a round shape (Fig. 1C). Stomatal guard cells were also enlarged (Fig. 1D).

When leaf fragments that included the intumescence areas and normal areas were fixed and embedded in Technovit 7100 resin and prepared as 2-μm-thick sections, we observed that resin peeled from the epidermal cells on the adaxial side where the cuticular layer had developed normally and from the normal area on the abaxial side (Fig. 2). However, the resin did not peel at the intumescence area where the cells were enlarged.
due to intumescence injury (Fig. 2). We observed that the epidermal cells and inner cells of the spongy tissue were enlarged and raised outward at the abbatial site. There seemed to be no increase in cell number, but there was an increase in cell volume. The volume of spongy tissue cells also increased compared with the normal area.

According to TEM observation, a cuticular layer developed in the epidermal cells on the abbatial side of normal leaves (Fig. 3A). The cuticular layer comprised a surface wax layer (cu1) at the top, an intermediate layer (cu2) with substantial cutting below it, and a lower layer (cu3) with a mixture of cutting and cell wall polysaccharides (cu3) (Dominguez et al., 2011, 2015; Heredia-Guerrero et al., 2014). However, the normal leaves were not clear, except for the boundary of the wax layer. On the other hand, abnormalities were observed in the cuticular layer on the surface of sites where intumescence injury occurred. First, it was clear that the outermost surface wax layer had disappeared in some places (Fig. 3C, arrows). In addition, the cuticular layer of normal cells was unclear between cu2 and cu3 layers; however, the cuticular layer became clear where bullous disease was apparent (Fig. 3D; cu2, cu3). Within the intermediate layer (Fig. 3E; cu2), electron density was lower than that of the intermediate layer, and a swollen portion was observed (Fig. 3E; *). In addition, an abnormal cuticular layer in which the intermediate layer and the polysaccharide were mixed together with the disappearance of the surface wax layer, became thinner than the cells with a normal cuticular layer (Fig. 3F, G).

**Experiment 2: Investigation of the leaf stage and conditions associated with intumescence**

When leaves with intumescence injury were stained with toluidine blue O aqueous solution, the intumescence area that appeared as a white enlargement was stained purple (Fig. 4A, B). It was possible to distinguish, by toluidine blue O staining, an intumescence area, where an abnormality had occurred in the cuticu-
lar layer, from a normal area, where the cuticular layer remained normal (Fig. 4C). Using this staining method, the stage at which intumescence injury occurred, the relationship between the water content of the leaf and the degree of intumescence, as well as the conditions required for the occurrence of intumescence injury, were investigated.

In order to investigate leaf stage sensitive to intumescence, tomato plants at different stages were placed in an incubator where the UV wavelength range was reduced, and the degree of intumescence on these plants was examined. Even after 4 days in an incubator, stained areas were not observed in young plants that had developed a cotyledon one week after sowing; thus, intumescence was not observed in these plants.

We examined the effects of low-UV treatment duration in an incubator on the intumescence degree of tomato leaves at different leaf positions at 14 and 21 days after sowing plants. Duration of low-UV treatment and leaf position significantly affected the intumescence degree of tomato leaves (Table 1). Significant interactions between duration of low UV treatment and leaf position were observed at 14 and 21 days after sowing plants, respectively. No intumescence was observed on any leaves one day after placing in an incubator under low UV condition. In contrast, intumescence injury occurred in plants with true leaves 14 or 21 days after sowing more than 2 days after they were placed in the incubator. In the plants placed in the incubator 14 days after sowing, slight intumescence injury occurred in the 1st to 3rd leaves during the 2 days in the incubator. However, the degree of intumescence increased with time in the incubator as intumescence injury occurred from the 1st to 4th leaves over 3 days, and from the 1st to 5th leaves over 4 days in the incubator. The degree of intumescences in the 1st and 2nd leaves were significantly higher than other leaves over 4 days in the incubator. On the other hand, intumescences were not observed in the cotyledon of these plants regardless of time.

In plants placed in the incubator 21 days after sowing, intumescence injury occurred in the 3rd to 6th leaves over 2 days in the incubator (Table 1). The extent of intumescence widened as the time in the incubator increased. The 3rd to 4th leaves died in plants placed in the incubator for 3 days, and the 2nd to 4th leaves died in plants placed in the incubator for 4 days. However, intumescence was not observed in the cotyledon or 1st leaf of plants placed in the incubator 21 days after sowing.

| Leaf position | Degree of intumescence (%) 14 days after sowing | Degree of intumescence (%) 21 days after sowing |
|---------------|-----------------------------------------------|-----------------------------------------------|
|               | Duration of low-UV treatment                  | Duration of low-UV treatment                  |
|               | 1 day 2 days 3 days 4 days                    | 1 day 2 days 3 days 4 days                    |
| Cotyledon     | 0 0 0 0                                        | 0 0 0 0                                        |
| 1st           | 0 0.2 67.2 100                                | 0 0 0 0                                        |
| 2nd           | 0 0.6 36.3 82.1                               | 0 0.6 66.7 100                                |
| 3rd           | 0 0.2 14.2 17.3                               | 0 3.5 100 100                                 |
| 4th           | 0 0 7.5 27.7                                  | 0 4 100 100                                   |
| 5th           | — — — 8.4                                     | 0 10.2 18.2 58.7                              |
| 6th           | — — —                                        | 0 3.2 10.4 53.3                               |
| 7th           | — — —                                        | 0 0.3 5.5 25                                  |

Duration of low-UV (A) ***z***
Leaf position (B) ***
A×B *

* and *** indicate significant at P<0.05 and P<0.001 by two-way ANOVA, respectively.
The degree of intumescence was higher in the upper leaves (the 5th leaf and above) of plants 21 days after sowing than in plants placed in the incubator 14 days after sowing. The above findings show that, intumescence injury occurred markedly in developing leaves that had expanded leaf areas; it also occurred over a period of 2 days under conditions of reduced UV and 85% relative humidity. It was found that the incidence of intumescence on tomato leaves increased with the duration of low UV, and sensitivity differed depending on stage.

In plants placed in the incubator for 3 days, 21 days after sowing, we investigated the degree of intumescence and changes in water content. The degree of intumescence and the water contents differed significantly depending on the UV conditions and leaf position (Table 2). Significant interactions between UV condition and leaf position were observed on the degree of intumescence and the water contents, respectively. The maximum degree of intumescence was about 50% in the 3rd leaf, and about 30% of total leaf area was affected by intumescence injury from the 2nd to 5th leaves. Intumescence was not observed in the cotyledons, and there was little occurrence in the 1st and 6th leaves. Leaf water content was generally reduced in plants with intumescence compared with normal plants (Table 2). Leaf water contents of 1st to 4th leaves were significantly decreased, in particular, the water content decreased in the 3rd and 4th leaves, where the degree of intumescence was high. Even in the 1st leaf of plants with intumescence, where the occurrence of intumescence injury was low, the water content was lower than in the control.

The relationship between RH and the occurrence of intumescence injury was investigated using plants placed in an incubator for 3 days, 17 days after sowing. The degree of intumescence changed as relative humidity changed, even though the light conditions remained constant and there was little UV radiation. When RH in the incubator was 90%, the degrees of intumescence in the 1st, 2nd, 3rd, and 4th leaves were about 80%, 40%, 8%, and 2%, respectively. At RH of 70%, the degree of intumescence was about 2%-3% (Table 3). Degree of intumescence at RH of 70% was significantly lower than at RH of 90% (Table 3). When RH was 60%, intumescence was not observed in the 4th leaf, and in the 2nd and 3rd leaves was about 1%.

### Discussion

In our cultivation study, when leaves with intumescence were embedded in Technovit 7100 resin and cut into ultrathin sections, the resin could be removed from the epidermal cells where the surface was not affected by intumescence injury; however, the resin did not peel from the enlarged epidermal cells affected by intumescence injury. Technovit 7100 resin is hydrophilic; thus, it was expected to peel because of the low affinity between the resin and cuticular wax. We speculate, therefore, that non-peeling in intumescence-affected areas

### Table 2. Degree of intumescence and water content of leaves at different positions on tomato plants under normal UV and low UV condition.

| Leaf position | Degree of intumescence (%) | Water content (%) |
|---------------|-----------------------------|------------------|
|               | Normal UV | Low UV | Normal UV | Low UV |
| 1st           | 0         | 1.3    | 91.2      | 89.1   |
| 2nd           | 0         | 26.3   | 91.0      | 88.7   |
| 3rd           | 0         | 44.3   | 91.7      | 83.4   |
| 4th           | 0         | 30.5   | 91.5      | 81.5   |
| 5th           | 0         | 26.1   | 90.1      | 86.4   |
| 6th           | 0         | 3.0    | 89.8      | 88.1   |

UV condition (A) *** NS, *** indicate significant $P < 0.001$ by two-way ANOVA.

Leaf position (B) *** NS, *** indicate significant $P < 0.001$ by two-way ANOVA.

A×B *** NS, *** indicate significant $P < 0.001$ by two-way ANOVA.

### Table 3. Degree of intumescence of leaves at different positions on tomato plants under 70% and 90% relative humidity conditions.

| Relative humidity | Leaf position | Degree of intumescence (%) |
|-------------------|---------------|-----------------------------|
|                   | 1st           | 0                           |
|                   | 2nd           | 2.2                         |
|                   | 3rd           | 2.8                         |
|                   | 4th           | 2.0                         |
| 70%               | 1st           | 77.3                        |
|                   | 2nd           | 34.2                        |
|                   | 3rd           | 8.5                         |
|                   | 4th           | 2.6                         |

Significance$^*$ Relative humidity ** NS, *** indicate significant $P < 0.001$ by two-way ANOVA.

Leaf position * NS, *** indicate significant $P < 0.001$ by two-way ANOVA.

Relative humidity × Leaf position * NS, *** indicate significant $P < 0.001$ by two-way ANOVA.

$^*$ ** and * indicate significant $P < 0.01$ and $P < 0.05$ by two-way ANOVA.
was caused by the disappearance of the cuticle wax on the surface or the loss of the water repellent function.

TEM observation revealed abnormalities in the cuticular layer of enlarged epidermal cells affected by intumescence injury. In particular, the outermost epicuticle wax disappeared, and abnormalities were observed in the cutting-containing layer under the epicuticle wax. In the enlarged cells, a decrease was observed. Toluidine blue O aqueous solution was used to identify the cuticular layer in mutants that cause cuticular abnormalities; it was shown that mutants were stained with toluidine blue O as well as individuals with cuticular abnormalities (Tanaka et al., 2004; Petit et al., 2014). Thus, enlarged cells due to intumescence injury can be associated with abnormalities in the surface cuticular layer. In previous studies on the morphology of intumescence, abnormalities in the cuticular layer were not demonstrated; however, in the present study, such abnormalities were the first finding. We found that these abnormalities were clearly distinguished by staining with toluidine blue O dye. Moreover, we revealed that the degree of intumescence in each leaf can be quantified using the staining ratio of toluidine blue O.

Transcriptome analysis comparing the gene expression profiles of leaf tissues with and without intumescences indicated that there were 1604 differentially expressed genes; these genes were involved in hormone response, DNA synthesis and repair, metabolic pathways, and cell wall biosynthesis (Waa et al., 2017). The authors of this study suggest that ethylene signaling may play a role in the occurrence of intumescence injury and that 3 beta-hydroxysteroid dehydrogenase may play a key role in UV inhibition of intumescence development (Waa et al., 2017). However, Waa et al. (2017) did not identify genes related to the cuticular layer in their study. It is possible that abnormalities in the cuticular layer are not a direct factor in the occurrence of intumescence injury. However, cuticular layer abnormalities are a clear morphological feature associated with intumescence injury; thus, they, and the factors that cause them, should be investigated further.

Many studies reported that intumescence injury occurs due to environmental factors, such as low UV and high relative humidity (Morrow and Tibbitts, 1988; Kubota et al., 2017; Miso et al., 2018). In our study, intumescence injury also occurred under low-UV and high-relative humidity conditions. The cuticular layer protects plants from UV and drought stress, and was reported to develop well under strong light conditions with high UV and under low relative humidity conditions (Stein Müller and Tevin, 1985; Cameron et al., 2006; Kosmas et al., 2009; Yeats and Rose, 2013; Lee and Suh, 2015). On the other hand, low UV and high relative humidity are considered conditions under which the cuticular layer has difficulty developing (Domínguez et al., 2011, 2015). We speculate that the development of the cuticular layer and the degree of intumescence are related.

The incidence of intumescence injury was examined in various leaf stages and found to be increased in developing leaves, but low in fully developed and young leaves with slow expanding speeds. Where the area of the leaf expands quickly, the formation of the surface cuticular layer also seems to occur rapidly. In such cases in developing leaves, inhibition of cuticular layer formation leads to easy induction of intumescence. We also found that water content was reduced in leaves with intumescence. This is apparently because the surface cuticular layer was damaged, and thus its ability to preserve moisture was affected. This suggests that the whole plant lost water, which would also increase damage.

In the present study, we showed that UV and high relative humidity cause intumescence and that the incidence of intumescence increases over time. In order to prevent intumescence in artificial conditions, plants should be irradiated with UV light, managed at low relative humidity, and setting in low UV and high relative humidity conditions for more than 2 days should be avoided. Strong UV and low relative humidity conditions may promote the formation of the cuticular layer. The high occurrence of intumescence and the weak development of the cuticular layer under low UV and high relative humidity conditions contrast with the lack of intumescence and well-developed cuticular layer observed under strong UV and low relative humidity conditions. Therefore, it is necessary to consider the development of the cuticular layer in order to reduce damage due to intumescence injury.

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