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Effect of grafting methods on physiological change of graft union formation in cucumber grafted onto bottle gourd rootstock

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ABSTRACT

To determine the effect of grafting method (hole insertion grafting, HIG; tongue approach grafting, TAG; and spliced grafting, SG) on graft union formation in cucumber grafted onto bottle gourd rootstock, an anatomical and physiological study was carried out during the healing stage. The results showed that the TAG method resulted in an obviously higher daily growth rate than the other grafting methods 7 days after grafting (DAG), with significantly higher values in the scion than in the rootstock. The isolation layer, callus formation, and vascular connection all occurred at the graft junction when using HIG, TAG, and SG. Vascular bridging first occurred between scion and rootstock using HIG or TAG at 5 DAG; this process was delayed with SG. The activities of superoxide dismutase (SOD), POD (peroxidase), CAT (catalase), PAL (Phenylalanine ammonia-lyase), and PPO (polyphenol oxidase) were significantly enhanced in the graft union during the healing process when different grafting methods were used. Compared with HIG and SG, the POD and CAT activities and total phenolic compounds were higher with TAG at 7 DAG; a reduction in PPO activity and lignin content were also overserved at the graft with TAG at 7 DAG. These results suggest that the association of TAG with a higher growth rate during the healing period is linked with the earlier improvement in vascular bundle connection that may be caused by increased antioxidant activities and lower lignin content at the graft junction. These morphological and physiological differences may provide valuable information for revealing the graft union healing process.

1. Introduction

Grafting is widespread in vegetables and woody crops where it helps to reduce crop damage resulting from soil-borne pathogens, improves production and quality, and increases plant abiotic stress tolerance (Savvas et al., 2010; Yang et al., 2012; Melnyk and Meyerowitz, 2015). Graft union formation is a critical event for successful grafting. Previous work describes three key events that occur at the graft junction: (I) ruptured cells collapse to form a necrotic layer, (II) cells proliferate to form callus, and (III) callus cells differentiate into vascular tissue to reconnect the phloem and xylem across the graft junction (Flaishman et al.,2008; Moore and Walker,1981; Jeffree and Yeoman, 1983). The mechanism of the biological process underlying graft union formation is, however, poorly understand.

Cell division is rapidly promoted during grafting in many plants (Asahina et al., 2002; Melnyk et al., 2015). Removing the cotyledons in cucumber inhibits cell division and prevents wound healing (Asahina et al., 2002). Cell differentiation into callus fills the gaps between adhering tissues (Aloni et al., 2003; Gardiner et al., 2010). When compared with cut but ungrafted Arabidopsis thaliana hypocotyls, cut and grafted hypocotyls produce little callus (Melnyk et al., 2015). Many studies have examined the induction of callus formation by wounding and have found that it is not a necessary requirement for successful junction formation (Melnyk, 2016). However, a functional vascular connection is a common theme for the establishment of a successful graft union (Turquois and Malone, 1996; Kester et al., 1997; Fernández-garcía et al., 2004). Recently, phloem connections were found to form 3 − 4 days after grafting (DAG) whereas xylem connections were found to form 6 − 8 DAG (Melnyk et al., 2015). Surprisingly, Arabidopsis scions grafted onto tomato rootstocks flower and produce seeds without scion/stock vascular connections (Flaishman et al., 2008). The graft junction connection process is species specific.

Complex physiological metabolites are affected during graft union formation (Koepke and Dhingra, 2013). Grafting, as a wounding stress,

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triggers antioxidant defense systems. Previous studies have indicated the presence of either a higher level of reactive oxygen species (ROS) or a less efficient detoxification system on incompatible scion/rootstock interfaces (Irisarri et al., 2015). SOD and CAT enzyme activities were higher in compatible than incompatible pear/quince unions and six antioxidant genes (SOD1, SOD3, APX3, APX6, CAT1, and CAT3) were differentially expressed between compatible and incompatible pear/quince unions. Higher ROS levels are associated with a failure to achieve adequate union between the graft partners at the early stages of development (Irisarri et al., 2015; Nocito et al., 2010). Hydrogen peroxide acts as a signal molecule in the hetero-grafting system (Wang et al., 2016) while certain antioxidases were also activated in the xylem lignifications of the newly differentiating vascular system during graft junction healing (Quiroga et al., 2000; Whetten et al., 1998). Furthermore, basal auxin transport into the rootstock may induce oxidative stress in grafted melon plants (Aloni et al., 2010) and non-enzymatic molecules such as flavonoids, ascorbic acid, and carotenoids may play a substantial role in counteracting oxidative stress (Mittler, 2002; Ashraf, 2009). Phenolic compounds, as part of a plant's defense mechanisms, were always synthesized at the graft interface (Errea et al., 1994; Pina et al., 2009). Phenolic acid and flavanol content can be used as chemical markers for the early detection of graft compatibility (Canas et al., 2015; Assunção et al., 2016). Phenols escape from the vacuole into the cytoplasmic matrix where they are oxidized by POD and PPO (Hartmann et al., 2002). Additionally, Pina and Errea (2008) demonstrated that the higher levels of PAL in incompatible Prunus unions led to an accumulation of phenol. Although many of the physiological changes associated with grafting have been established, information regarding graft union healing remains limited.

In vegetables, hole insertion grafting (HIG), tongue insertion grafting (TAG), and splice grafting (SG) are the most common grafting methods (Lee et al., 2010). In cucumber, HIG and TAG are the most popular, while SG is the easiest for commercial production (Huang et al., 2015; Lee et al., 2010). To our knowledge, no studies have been conducted to investigate the effect of different grafting methods on the graft interface healing process. The main goals of the present study were (1) to characterize the anatomical developmental stages of graft union formation of different grafting methods in cucumber/pumpkin and (2) analyze the physiological changes in the graft interface among different grafting methods to provide valuable information relating to the graft union healing process.

2. Materials and methods

2.1. Plant materials and growth conditions

The study was conducted at the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, China. A cucumber cultivar (*Cucumis sativus* L. 'Zhongnong NO.26') was used as the scion, a pumpkin cultivar (*Cucurbita moschata* 'Jingyan NO.5') was used as the rootstock. Seeds of the scions and rootstocks were respectively sown in 50-cell and 32-cell polystyrene trays containing commercial organic substrates ($V_{peatmoss}$:$V_{vermiculite}$:$V_{perlite}$ = 1:1:1). The germination environmental conditions were 25–28℃ and 85–90% relative humidity. Grafting was conducted using the three different grafting methods described below (Fig. S1).

HIG: rootstocks were sown 2–3 d earlier than scions (6–7 d after sowing). When cotyledons of the scion were fully opened and the first true leaf of the rootstock started to develop (9–10 d after sowing) plants grafted as previously described (Mohamed et al., 2014).

TAG: scions were sown 5 d earlier than rootstocks. After the scion had fully developed the first true leaf (14–15 d after sowing) and the rootstock had started to develop the first true leaf (9–10 d after sowing) TAG was performed as described previously (Mohamed et al., 2014).

SG: scions were sown 2 d earlier than rootstocks. When cotyledons of the scion had fully opened (6 d after sowing) and the cotyledon of the rootstock had started to open (4 d after sowing), cotyledon and growing point of rootstocks were removed and a 30° angled cut was made on the hypocotyl of the rootstocks and scion with a razor blade. The scion hypocotyl was then spliced to the hypocotyl of the rootstock using a plastic tube as previously described (Lee et al., 2010).

Grafted plants were transferred to a humidity chamber where they were maintained at a temperature of 26/20 °C day/night with a relative humidity of 80–95% for about 7 d during graft union healing. For the first 3 d the environment was kept dark using shading; from 4 to 5 DAG the plants were grown in light of 130–150 μmol m^{-2} s^{-1} with 90–95% humidity and from 6 to 7 DAG the light was increased to approximately 300 μmol m^{-2} s^{-1} with 80–90% humidity.

The three different grafting treatments were performed simultaneously with 100 plants per grafting method; all grafts were performed by one operator. The plants were irrigated with 1/2 Hoagland nutrient solution. Seven DAG the grafted plants and graft union were examined and the following parameters measured.

2.2. Histology of graft development

Changes in the histology of heterografts of cucumber on pumpkin generated using different grafting methods were examined by harvesting eight graft unions per time point at 1, 3, 5, and 7 DAG. Samples were trimmed to between 3 mm above and 3 mm below the graft junction (Fig. S2), and fixed for 2 d in FAA before being dehydrated through an ethanol/xylene series followed by a xylene/paraplast series, embedded in paraplast, and sectioned as described previously (Takacs et al., 2012). After samples were sectioned to 10 μm vertically using a rotary microtome (KE3390; KEDEE; Zhejiang, China), dewaxed, rehydrated, and cleaned they were stained with Fast Green, counterstained with Safranin, and fixed with Neutral Balata. Sections were examined using a light microscope (BX53; Olympus Corp., Tokyo, Japan) and representative sections were photographed. We use 5 replicates per time point to observe the structure (Table S1).

2.3. Determination of biomass

The scions (shoot above the graft union) and rootstocks (the part below the graft union) harvested at 0 and 7 DAG were washed using deionized water and dried with tissue paper. The fresh weight of each scion and rootstock was determined by weighing. The samples were then placed into an oven at 105 °C for 15 min before being held at 75 °C until dried. The weight of each sample was then measured again. The daily growth rate was calculated as follow, Absolute Growth Rate (AGR) = (W_{7d}-W_{0d})/ (T_{7d}-T_{0d}), W_{7d}, weight of biomass at 7 DAG; W_{0d}, weight of biomass at 0 DAG, T_{7d}, 7 DAG; T_{0d}, 0 DAG (Baligar et al., 1993). The mean of 14 plants was obtained for each time point.

2.4. Determination of SOD, POD, and CAT activities

The graft unions of plants obtained using different grafting methods were excised 0, 1, 3, 5, and 7 DAG and rapidly weighed. For each sample, 0.5 g fresh weight of tissue was immediately ground with a pestle in an ice-cold mortar with 4 ml 50 mM phosphate buffer (pH 7.0). The homogenates were centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatant was used to measure enzyme activities (Mishra et al., 2006). SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium. POD activity was measured as the increase in absorbance at 470 nm caused by guaiacol oxidation (Polle et al., 1994). CAT activity was measured as the decline in absorbance at 240 nm caused by a decrease in H_2O_2 removal (Rao et al.,1996).

2.5. Determination of PAL and PPO activities

Graft union samples (0.5 g fresh weight) that were harvested at 0, 1,