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Short communication

Experimental evidence for benefit of self discrimination in roots of a clonal plant

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Abstract. Some plants express self discrimination in their roots, which allows them to preferentially reduce antagonistic interactions and increase facilitative interactions with genetically identical ramets or individuals. However, our understanding of how self discrimination contributes to reproduction in plants is limited. Here, we report that self discrimination is adaptive in the clonal plant *Kalanchoë daigremontiana*. Plants were grown with a self or non-self plant for 30 days. Furthermore, root allocation patterns associated with self and non-self plants and their root exudates were investigated in a split-root experiment. Biomass of shoot, root, and plantlets and number of plantlets per plant were examined. Plants expressed root behaviour that is specific to the non-self condition: plants developed more roots when growing with a non-self plant than when growing with a self plant. Similar root behaviour was observed in the root exudate experiment, in which it reduced both root growth and clonal reproduction in non-self competitor plants. As a result, plants competing with a clonal self plant produced more clonal plantlets than plants competing with a non-self plant. These experimental results provide evidence that self discrimination through root exudation is adaptive for *K. daigremontiana* plants.

Keywords: Clonal plant; intraspecific competition; invasive species; population growth; root behaviour.

Introduction

Self discrimination is a taxonomically widespread phenomenon. In animals, self discrimination has been well documented and many types have been identified, including immune system discrimination in vertebrates, incompatibility in somatic fusion experiments and self-incompatibility between eggs and sperm in ascidians (e.g. Burnet 1971; Medzhitov and Janeway 2002). In plants, owing to their limited dispersal, interacting individuals are often close genealogical relatives (Argyres and Schmitt 1991; Vekemans and Hardy 2004). For example, the most well-known form of self discrimination is self-incompatibility, a widespread mechanism in flowering plants that distinguishes between self and non-self pollen to prevent self-fertilization (Nasrallah 2002). Thus, self discrimination plays important roles in biological systems.

Recent studies found another type of self discrimination in roots. Some plants can change their root allocation and morphology in response to the presence or absence of non-self neighbours as a result of root discrimination mechanisms (Mahall and Callaway

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1991, 1996; Falik *et al.* 2003; Holzapfel and Alpert 2003; Gruntman and Novoplansky 2004). For example, when in contact with self, plants develop fewer and shorter roots than when in contact with non-self individuals (Falik *et al.* 2003; Holzapfel and Alpert 2003; Gruntman and Novoplansky 2004; but see Hess and De Kroon 2007). Such root discrimination is thought to be mediated by environmentally based physiological coordination rather than by genotype-specific differences, as seen in self-incompatibility and immune systems (Mahall and Callaway 1991, 1996; Falik *et al.* 2003; Holzapfel and Alpert 2003; Gruntman and Novoplansky 2004; Fukano and Yamawo 2015). Self discrimination in roots may be an adaptation to minimize wasteful competition with self (Novoplansky 2009) by changing the root growth direction and spatial pattern of below-ground parts (Herben and Novoplansky 2008).

A clonal plant also expresses self discrimination that is not mediated by environmentally based physiological coordination. Karban and Shiojiri (2009) reported that sagebrush plants that received volatile cues from genetically identical cuttings accumulated less natural damage than those that received cues from non-self cuttings. Such self discrimination that does not require physical coordination may be found in the root system, and it should have adaptive functions. However, our understanding of how self discrimination contributes to reproduction in clonal plants is limited. In this study, we provide experimental evidence that self discrimination is adaptive in clonal reproduction.

To examine the effects of the ability to self-discriminate on clonal reproduction, we investigated *Kalanchoë daigremontiana*, a common invasive clonal plant (Herrera and Nassar 2009; Randall 2012; Wang *et al.* 2016). This species reproduces both sexually and clonally. It asexually produces many plantlets that grow on leaf margins, and these plantlets detach, drop to the ground and grow near their mother plant, where they are likely to meet both self and non-self plants. If plants avoid competition through self discrimination, we would expect plants growing with self (clonal sibling) plants to invest more in reproduction, such as clonal reproduction by plantlets, than in plants growing with non-self plants (Table 1). In addition, we would expect differences in clonal reproduction related to the root allocation pattern in response to the genotype of neighbouring plants. To detect self discrimination in *K. daigremontiana*, we conducted a root exudate experiment, comparing the root allocation response to different root exudates, self or non-self. If self discrimination is mediated by root exudates, we would expect the root allocation to differ between self and non-self conditions.

Methods

Cultivation of mother plants

One plantlet per *K. daigremontiana* plant was collected from two plants from Saga City on Kyushu Island ($n = 2$) and from Naha City on Okinawa Island ($n = 2$), respectively, in October 2012. To produce mother plants, in November 2012, each plantlet was planted in a plastic pot (5 × 5 × 10 cm) containing garden soil (Sun & Hope Co., Japan). These pots were maintained in a growth chamber at 25 °C under a 12L:12D photoperiod for 6 months until many plantlets had grown on the leaf margins. Water was provided every other day.

Competition experiment

In April 2013, 11 or 8 clonal plantlets were collected from each mother plant. Two plantlets were planted in a plastic pot (5 × 5 × 10 cm) containing garden soil (Sun & Hope Co.), at a distance of ~2 cm from each other, and placed in a growth chamber at 25 °C under a 12L:12D photoperiod for 30 days. Target plants were grown with either a clonal plantlet originating from the same mother plant ($n = 10$) as the self condition or from a

Table 1. Questions and predictions of each experiment.

Experiment	Question	Predictions
Competition experiment	Do plants avoid competition with self plants?	Plants produce more plantlets in the self condition than in the non-self condition.
Split-root experiment	Are the differences in clonal reproduction related to root behaviour in response to the genotype of neighbouring plants?	Plants growing with non-self plants express competitive behaviour, such as increasing root allocation towards non-sibling plants (i.e. root biomass is larger in the Falcon tube with a non-sibling plant).
Root exudation experiment	Do plants discriminate self versus non-self plants through root exudates?	Plants allocate more roots in the Falcon tube with root exudate of a non-self plant.

different mother plant (n = 10) as the non-self condition **[see Supporting Information—Table S1]**. After 30 days, we counted the number of clonal plantlets on each plant. The plants were then harvested and divided into roots, shoots and clonal plantlets on leaves, which were weighed to 10^{-4} g on an electronic balance (BP210D; Sartorius AG, Göttingen, Germany).

Split-root experiment

To reveal the root behaviour in response to self versus non-self discrimination, we conducted a split-root experiment, a commonly used approach to investigate root behaviour (O'Brien *et al.* 2005). We collected 10 clonal plantlets from each mother plant and planted one per plastic pot (5 × 5 × 10 cm) containing garden soil (Sun & Hope Co.) in a growth chamber at 25 °C under a 12L:12D photoperiod for 30 days. Water was provided every other day. During this period, the plants grew to ~5 cm in height and had eight leaves. These 40 plants were assigned per genotype as target and competitor plants, respectively.

Each plant was removed from the pot and its roots were washed with distilled water. One portion of the plant's roots was placed in a 50-mL Falcon tube containing only sand, and the other portion was placed in an identical tube with sand and a self or non-self plant (self, n = 20; non-self, n = 20; **see Supporting Information—Table S2, Fig. S1**). We applied 15 mL of water to each tube every other day. After 30 days, we counted the number of clonal plantlets on each plant. All plants were harvested and dried at 80 °C for 3 days. Then, they were divided into roots, shoots and clonal plantlets produced on leaves. The dried roots in each tube, shoots and clonal plantlets were weighed to 10^{-4} g on an electronic balance.

Root exudation experiment

In January 2017, 25 clonal plantlets were collected from each of four mother plants and planted individually in plastic pots (5 × 5 × 10 cm) containing sand in a growth chamber at 25 °C under a 12L:12D photoperiod for 30 days. Water was provided every other day. During this period, the plants grew to ~5 cm in height and had eight leaves. Ten and 15 plants per genotype (for a total of 40 and 60 plants) were used for root exudate collection and root exudate application, respectively.

Root exudate collection. To investigate the role of root exudation in self versus non-self discrimination and root behaviour, we conducted a root exudation experiment. Root exudates were collected within the water that leached through soil occupied by plants of different genotypes. We applied 15 mL of distilled water to each pot and collected the root exudate solution that leached through. The root exudate solution was collected just prior to root exudate application. When the root exudate solution was <15 mL, distilled water was added to bring the volume to 15 mL.

Root exudate application. Plants were removed from the pots and the roots were washed with distilled water. Each plant's roots were divided in half, and each half was placed in a 50-mL Falcon tube containing sand **[see Supporting Information—Fig. S2]**. Five plants per genotype (total of 20 plants) were assessed in each of the three experimental conditions: (i) 15 mL of distilled water in one tube versus 15 mL of distilled water in the other tube (control); (ii) 15 mL of distilled water versus 15 mL of self root exudate solution (self); and (iii) 15 mL of distilled water versus 15 mL of non-self root exudate solution (non-self) **[see Supporting Information—Table S2]**. The distilled water and root exudate solutions were provided every other day.

After 30 days, we counted the number of clonal plantlets on each plant. The plants were harvested and dried at 80 °C for 3 days. Then, they were divided into roots, shoots and clonal plantlets produced on leaves, which were weighed to 10^{-4} g using an electronic balance.

Data analysis

Statistical analyses were performed in R v. 2.15.1 software (R Development Core Team 2012). Growth characteristics (i.e. the shoot, root, and total biomasses and biomass allocation, which reflects the interaction among root and shoot biomasses and treatment) were compared between the self and non-self conditions using generalized linear mixed models (GLMMs) with a Gaussian distribution and identity link function, including the genotype (parent plant ID) as a random effect. The clonal reproductive characteristics (i.e. number of plantlets per plant, plantlet biomass per plant and individual plantlet biomass) were also compared between the two conditions using GLMM with a Gaussian distribution and identity link function, including the genotype (parent plant ID) as a random effect. We conducted the F-test in the GLMM analyses. The relationships between number of plantlets and root biomass was analysed using a GLMM with a Gaussian distribution and an identity link function, including the genotype (parent plant ID) as a random effect. Shoot biomass was included as an explanatory variable as a covariance factor. The root biomass between the two Falcon tubes was analysed by GLMMs with a Gaussian distribution and identity link function, including the genotype (parent plant ID) and individual ID as random effects. Experimental conditions (i.e. treatment of each tube and their interactions) were included as explanatory variables. When the interaction was significant, we analysed the effects of treatment on the root biomass by GLMMs with a Gaussian distribution and identity link function, including the genotype (parent plant ID) and individual ID as random effects. The relationship between the root biomass in Falcon tube with competitor of the target plant and root biomass of the competitor was analysed using a GLMM with a Gaussian distribution with an identity link function, including the genotype as a random effect.

Results

Competition experiment

After 30 days of culture, the shoot, root and total biomasses did not differ between the self and non-self conditions (Table 2). Biomass allocation to root and shoot also did not differ significantly between treatments ($F = 1.82$, $P = 0.19$). The number of clonal plantlets produced per plant under the self condition was higher than that under the non-self condition (Table 2). The number of clonal plantlets per plant was positively correlated with the root biomass in both conditions (GLMM; $P = 0.002$; estimated coefficient = 35.37). However, the total biomass of clonal plantlets per plant and biomass of a clonal plantlet did not differ between the two conditions.

Split-root experiment

The shoot, root, and total biomasses and the reproductive traits (number of plantlets, biomass of plantlets per plant and biomass of a plantlet of target plants) did not differ among experimental conditions (Table 3). Likewise, biomass allocation did not differ among

Table 2. Growth and clonal reproductive traits of *Kalanchoë daigremontiana* in the competition experiment. Forty plantlets originating from four mother plants were used in the experiment.

Plant characteristics	*n*	Conditions		*F*-value	*P*-value[1]
		Self	Non-self		
Growth characteristics					
Shoot (g)	10	9.4 ± 2.8	10.4 ± 2.0	0.99	0.461
Root (g)	10	0.4 ± 0.1	0.3 ± 0.1	4.93	0.086
Total (g)	10	10.0 ± 3.0	10.5 ± 2.3	0.61	0.612
Clonal reproductive characteristics					
No. of plantlets	10	22.7 ± 5.8	13.9 ± 7.7	25.36	**<0.01**
Dry weight of plantlets/plant	10	1.0 ± 0.3	0.7 ± 0.5	3.54	0.128
Dry weight of a plantlet	10	0.05 ± 0.01	0.05 ± 0.02	0.27	0.612

[1]*P*-values were corrected using the false discovery rate.

Bold indicates $P < 0.05$.

Table 3. Growth and clonal reproductive traits of *Kalanchoë daigremontiana* in the split root experiment. Forty target plantlets and 40 competitor plantlets originating from four mother plants were used for the experiment.

Plant characteristics	Target plant				Competitor plant			
	Condition		*F*-value	*P*-value[1]	Condition		*F*-value	*P*-value[1]
	Self	Non-self			Self	Non-self		
Growth characteristics								
Shoot biomass (g)	6.1 ± 1.0	5.5 ± 1.0	2.61	0.20	6.2 ± 1.3	6.2 ± 1.0	0.0091	0.92
Root biomass (g)[2]	0.3 ± 0.07	0.2 ± 0.07	4.38	0.20	0.4 ± 0.10	0.3 ± 0.09	12.837	**0.003**
Total biomass (g)	6.8 ± 1.6	5.7 ± 1.1	2.95	0.20	6.6 ± 1.4	6.5 ± 1.1	0.037	0.92
Clonal reproductive characteristics								
No. of plantlets/plant	23.4 ± 5.2	18.6 ± 4.1	10.8	**<0.01**	21.8 ± 4.7	18.1 ± 5.4	5.31	0.063
Dry weight of plantlets/plant (g)	0.2 ± 0.1	0.1 ± 0.1	6.45	**0.02**	0.2 ± 0.1	0.1 ± 0.1	4.37	0.078
Dry weight of a plantlet (g)	0.01 ± 0.004	0.01 ± 0.003	0.06	0.80	0.01 ± 0.004	0.01 ± 0.004	0.03	0.92

[1]*P*-values were corrected using the false discovery rate.

[2]Root biomass indicates the total of the divided roots.

Bold indicates $P < 0.05$.

conditions in both target (GLMM, $F = 2.003$, $P = 0.166$) and competitor plants (GLMM, $F = 3.89$, $P = 0.056$). On the other hand, root biomass in the two Falcon tubes did differ among experimental conditions (GLMM, $F = 2.01$, $P = 0.025$; Fig. 1). In the self condition, similar root biomasses were allocated to the two Falcon tubes (GLMM, $F = 74$, $P = 0.712$), whereas in the non-self condition plants allocated more root biomass to the Falcon tube with the competitor (GLMM, $F = 0.35$, $P = 0.049$).

Shoot and total biomasses of competitor plants did not differ between the self and non-self conditions (Table 3). However, root biomass was significantly smaller and higher, respectively, in plants growing with non-self plants. The root biomass of competitor plants was negatively correlated with root biomass of target plants inside the competitor plant tube in both conditions (GLMM, estimate coefficient = −0.77, $F = 5.58$, $P = 0.02$). The number of clonal plantlets per plant was positively correlated with the root biomass in both conditions (GLMM; $P = 0.003$; estimated coefficient = 136.1).

Root exudation experiment

There were no significant differences in the growth or clonal reproductive characteristics among the control, self and non-self treatments in the root exudation experiment (Table 4). Biomass allocation did differ among experimental conditions: in self and non-self conditions, shoot biomass was positively correlated with root biomass ($P = 0.023$), but in the control, it was not ($P = 0.61$). Root biomass in the two Falcon tubes also differed among conditions (Fig. 2): in the control and self root exudate conditions, similar root biomasses were allocated to the two tubes. On the other hand, in the non-self root exudate condition, plants allocated more root biomass to the tube with non-self exudate.

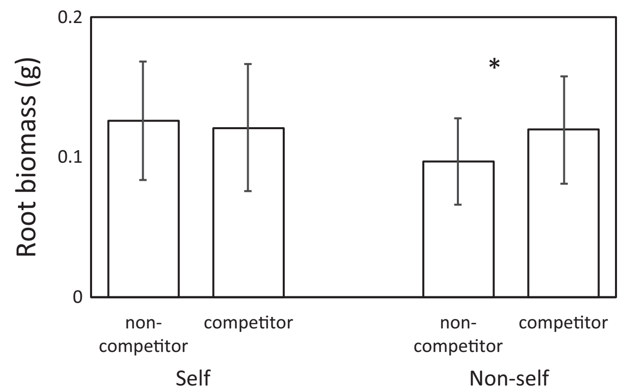

Figure 1. Root distribution of *Kalanchoë daigremontiana* against self ($n = 20$) or non-self ($n = 20$) competitor plants in the split-root experiment. Error bars represent the standard deviation. *Significantly different (GLMM, $P < 0.05$).

Discussion

The adaptive significance of self versus non-self discrimination and its consequences for competition have been discussed (Gruntman and Novoplansky 2004; Hess and De Kroon 2007). Our experiments clearly demonstrated that plants competing with clonal sibling plants invested more in clonal reproduction as compared to plants competing with non-siblings. These results provide evidence that self versus non-self discrimination is adaptive for *K. daigremontiana* plants.

Several studies reported that plants make greater root investments when competing with stranger plants (e.g. Dudley and File 2007; Biedrzycki and Bais 2010). Our experiments also demonstrated this trait in *K. daigremontiana* (Fig. 1), and this species significantly inhibited root growth of non-self competitor plants (Table 3). However, *K. daigremontiana* plants did not change their root allocation

Table 4. Growth and clonal reproductive traits of *Kalanchoë daigremontiana* in the root exudation experiment. Sixty plantlets originating from four mother plants were used as target plants, and 40 plantlets originating from four mother plants were used for root exudate collection.

Plant characteristics	Condition			*F*-value	*P*-value[1]
	Control	Self	Non-self		
Growth characteristics					
Shoot biomass (g)	6.0 ± 1.0	6.5 ± 1.5	5.7 ± 1.3	2.1	0.33
Root biomass (g)[2]	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.32	0.84
Total biomass (g)	6.2 ± 1.0	6.8 ± 1.6	6.0 ± 1.4	2.03	0.33
Clonal reproductive characteristics					
No. of plantlets/plant	21.8 ± 4.7	19.5 ± 6.0	17.3 ± 5.5	3.35	0.30
Dry weight of plantlets/plant (g)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.17	0.84
Dry weight of a plantlet (g)	0.01 ± 0.01	0.01 ± 0.003	0.01 ± 0.01	0.55	0.81

[1]*P*-values were corrected using the false discovery rate.

[2]Root biomass indicates the total of the divided roots.

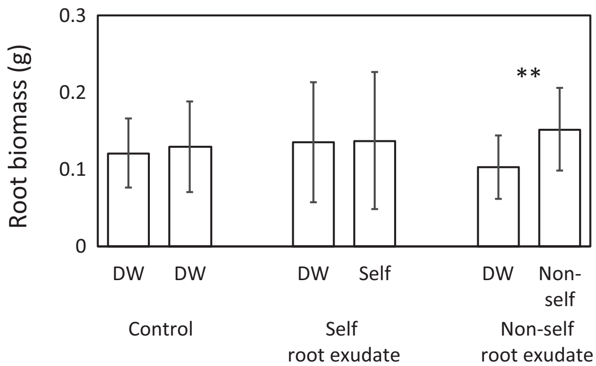

Figure 2. Root allocation of *Kalanchoë daigremontiana* in the root exudation experiment. **Significantly different (GLMM, $P < 0.001$). DW, distilled water.

against clonal self plants. Therefore, plants growing with self plants can develop their roots while avoiding root competition. Clonal reproduction was positively correlated with root allocation in both competition conditions. Thus, self versus non-self discrimination may minimize the competition between clonal self plants and achieve optimal root distribution and greater clonal reproduction. Further study is required to reveal the relationships between root behaviour and competition mechanisms.

Previous studies in other species reported that plants can discriminate self from non-self and kin from non-kin individuals in their neighbourhood by using chemical cues received by their above-ground tissues (Karban and Shiojiri 2009; Karban *et al.* 2013). Below-ground tissues express self discrimination via root exudation (Biedrzycki and Bais 2010). Our experiments indicated that *K. daigremontiana* discriminates self versus non-self plants using root exudation. On the other hand, most reported occurrences of self discrimination were mediated by physiological coordination (connection) between plants (Falik *et al.* 2003; Holzapfel and Alpert 2003; Gruntman and Novoplansky 2004; Fukano and Yamawo 2015); self discrimination in *K. daigremontiana* did not require the physiological coordination as observed in kin discrimination (Dudley and File 2007; Biedrzycki and Bais 2010). These results suggest that the mechanism of self discrimination in *K. daigremontiana* plants may be similar to the mechanisms of kin discrimination in other plant species, and it allows altruism towards relatives (Table 2), as does kin discrimination (e.g. Dudley and File 2007; Biedrzycki and Bais 2010).

It is unclear whether non-self plants were in fact genetically different clones. However, our experiments removed differences in environmental conditions such as maternal effects, because all the mother plants were cultivated under the same conditions in a growth chamber. Our interpretation that self discrimination among plants from the same mother plant was achieved to increase clonal reproduction was not affected by whether or not some of the non-self pairs belonged to genetically severed self.

Many invasive plant species colonize with numerous individuals and overrun native species. *Kalanchoë daigremontiana* is an invasive species in many southern regions (Herrera and Nassar 2009; Wang *et al.* 2016). Our findings suggest that the population growth of *K. daigremontiana* through clonal reproduction depends on the local spatial-genetic structure. Invasive species often have low genetic diversity due to the bottleneck effect (Lee 2002). Self discrimination in clonal populations may play an important role in population growth through its enhancement of reproduction in each individual (Herben and Novoplansky 2008), especially in introduced regions. Future studies concerning the relationships between self or kin discrimination and biological invasion are needed.

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Contributions by the Authors

A.Y. and H.M. developed the core idea and designed the experiments. A.Y., H.M. and S.M. carried out all experiments. A.Y. performed data analyses. A.Y. and H.M. wrote the article.

Conflicts of Interest

None declared.

Supporting Information

The following additional information is available in the online version of this article—

Table S1. Overview of the experimental design in non-self conditions in the competition experiment.

Table S2. Overview of the experimental design in on-self conditions.

Fig. S1. Experimental setups of *Kalanchoë daigremontiana* divided root competition experiment.

Fig. S2. Experimental setups of *Kalanchoë daigremontiana* root exudate experiment.

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