DR. YUCHAN ZHOU (Orcid ID : 0000-0001-8218-7603)

Article type : Research Paper

handling Editor: Prof. G Thiel

Plasma membrane H^+-ATPase activity and graft success of breadfruit (*Artocarpus altilis*) onto interspecific rootstocks of marang (*A. odoratissimus*) and pedalai (*A. sericicarpus*)

Yuchan Zhou[1, 2*] **and Steven J. R. Underhill**[1, 2]

[1]Queensland Alliance for Agriculture and Food Innovation, University of Queensland, St Lucia, QLD 4072, Australia

[2]Faculty of Science, Education and Engineering, University of the Sunshine Coast, Sippy Downs, QLD 4556, Australia

*Correspondence: Yuchan Zhou, e-mail: y.zhou1@uq.edu.au

Abstract

- Breadfruit (*Artocarpus altilis*) is primarily grown as a staple tree crop for food security in the Oceania. Significant wind damage has driven an interest in developing its dwarfing rootstocks. Due to the predominantly vegetative propagation of the species, grafting onto interspecific seedlings is an approach to identifying dwarfing rootstocks. However, grafting of breadfruit onto un-related *Artocarpus* species has not been investigated.
- Here we first reported the success of breadfruit grafting onto interspecific rootstocks, marang (*A. odoratissimus*) and pedalai (*A. sericicarpus*). To address the low graft survival, we investigated the relationship of plasma membrane (PM) H^+-ATPase activity to graft success.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/plb.12879

- We provided the first evidences for a positive correlation between PM H^+-ATPase activity and graft survival. The graft unions of successful grafts had higher PM H^+-ATPase activity compared to those of failed grafts. Rootstocks with low PM H^+-ATPase activity in leaf microsomes before grafting had lower graft survival than those with high enzyme activity, with graft success of 10% vs 60% and 0% vs 30% for marang and pedalai rootstocks respectively. There was a positive correlation between graft success and the PM H^+-ATPase activities measured from the rootstock stem microsomes two months after grafting (marang, r(7) = 0.9203, P= 0.0004; pedalai (r(7) = 0. 8820, P = 0.0017). Removal of scion's own roots decreased the leaf PM H^+-ATPase activity of grafted plants regardless of the final graft outcome. The recovery of the enzyme activity was only found in the successful grafts.
- The function of PM H^+-ATPase in graft union development and graft success improvement is discussed.

Keywords Breadfruit (*Artocarpus altilis*); grafting; plasmas membrane H^+-ATPase; marang (*A. odoratissimus*); pedalai (*A. sericicarpus*); rootstock; scion

1. Introduction

Breadfruit [*Artocarpus altilis* (Parkinson) Fosberg)] is a traditional fruit trees throughout the tropics. The species comprises fertile and sterile diploids (2n=2x = 56) and sterile triploids (2n = 3x = 84), and has hundreds of cultivars, with great diversity in morphological and agronomic characteristics (Ragone, 1997; Ragone, 2006). The species is primarily grown as a staple crop for food security in the Oceania. However, being an evergreen tree from 15-30 m, breadfruit is prone to wind damage. Significant tree loss due to intense tropical windstorm has driven an increasing interest in developing dwarf breadfruit varieties (Daley et al., 2012; Roberts-Nkrumah, 2012; Zhou et al., 2014). Dwarfism has been achieved by the widespread use of dwarfing rootstocks in many other fruit tree species; however, dwarfing rootstock for breadfruit has not been investigated. Vegetative propagation is required for the seedless varieties of breadfruit and preferred for the seeded varieties due to their recalcitrant seeds (Ragone, 1997; Ragone, 2006). Clonal propagation of breadfruit is generally through root suckers, root cuttings, or air layering. Breadfruit grafting has been practised, but not as a main propagation method (Solomon and Roberts-Nkrumah, 2008). There is limited information regarding rootstock properties and the performance of grafted breadfruit cultivars. In addition, vegetatively propagated rootstocks may not be

ideal for wind resistance due to their shallow root system (Solomon and Roberts-Nkrumah, 2008; Roberts-Nkrumah, 2012). Dwarfing rootstocks of breadfruit may come from other seeded species within the *Artocarpus* genus. The *Artocarpus* is a genus of approximately 60 trees and shrubs, including *A. camansi* (breadnut), *A. mariannensis* (dugdug), *A. odoratissimus* (marang) and *A. sericicarpus* (pedalai) (Zerega et al., 2010). Breadfruit interspecific grafting has been reported only on *A. camansi* and *A. mariannensis* rootstocks (Nandwani and Kuniyuki, 2005; Medagoda and Chandrarathna, 2007; Solomon and Roberts-Nkrumah, 2008). Species *A. camansi* and *A. mariannensis* are closely related to breadfruit (*A. altilis*) with breadfruit being domesticated from *A. camansi,* and the occurrence of introgressive hybridization between *A. altilis* and *A. mariannensis* (Zerega et al., 2004). Even though, poor survival has been reported on breadfruit grafting onto these rootstocks. Solomon and Roberts-Nkrumah (2008) reported an 18~28% of scion survival on *A. camansi* rootstock 6 weeks after grafting. They found neither grafting techniques (side graft, top wedge, whip and tongue) nor rootstock age had significant effect on graft success (Solomon and Roberts-Nkrumah, 2008). On the other hand, Nandwani and Kuniyuki (2005) found granting on seedy *A. mariannensis* could be improved to over 60% by approach grafting. Clearly, to achieve large-scale grafting and screening for dwarfing properties, a breadfruit grafting protocol with high graft success for interspecific rootstocks is a prerequisite.

Graft success of tropical fruit trees may be improved when a number of factors are considered. These generally include grafting techniques, scion/rootstock age and environmental factors. These factors have been linked to the capacity and favourable condition for cambium cell division and differentiation (Melnyk, 2017). Accumulating evidences have identified wound response, hormone signalling, callus formation and differentiation as the main events during graft union formation (Yin et al., 2012; Melnyk and Meyerowitz, 2015). However, candidates involved in these events are less clear. In plants, plasma membrane (PM) H^+-ATPase plays a central role in nutrient transport (Palmgren, 2001). The enzyme generates the proton-motive force that drives the uptake of nutrients such as sugars and ions across the plasma membrane of growing cells. This process is essential for physiological responses, including cambial cell division and expansion, vascular bundle differentiation, phloem loading and stress adaptation (Fromm et al., 1989; Michelet and Boutry, 1995; Palmgren, 2001). Increase in PM H^+-ATPase has been predominantly found in active cambial zone, xylem and phloem parenchyma, and stomatal guard cells (Pavlovkin et al., 2002; Arend et al., 2004; Paiva et al., 2008). To our knowledge, there is little information on a possible correlation between level of PM H^+-ATPase activity and graft survival. The knowledge may provide opportunity to develop strategies for fast screening of rootstocks and scions for prediction and improvement of graft survival for breadfruit species.

In this study, we reported the success of breadfruit grafting onto interspecific rootstocks, marang and pedalai. In order to identify factors causing the low graft success, we examined the levels of PM H^+-ATPase activity in graft union, rootstock and scion microsomal fractions and their relationship to graft success. For the first time, we provided evidences for a positive relationship between PM H^+-ATPase activity and the survival of grafted plants.

2. Materials and methods

2.1. Plant materials and treatments

Breadfruit (*Artocarpus altilis*, cultivar Cannonball), marang (*Artocarpus odoratissimus*) and pedalai (*Artocarpus sericicarpus*) were obtained from a commercial nursery at Cairns, northern Queensland. Breadfruit plants from root cuttings and, marang and pedalai plants as seedlings, were grown under glasshouse condition at 25 ~ 28 °C with natural daylight and daily water supply. Plants were grown in pots containing vermiculite and soil mixture as described previously (Zhou and Underhill, 2016). For grafting experiment, scions were selected from young plants 30 ~ 50 cm tall and rootstocks were seedlings of 2 to 3 months old. Each rootstock was paired randomly with the scion based on their similar stem thickness. Grafting was performed through approach graft with modification for *Artocarpus* species. In this technique, both the eventual scion and rootstock are self-sustained by their own roots while the graft heals to increase graft success. Briefly, to start the grafting, both rootstock and scion were brought next to each other, at the point where the graft union was to occur, leaves were removed, and a slice 3 to 5 cm long and 40% thickness of the stem in depth was removed from each stem. The two peeled surfaces were then bound tightly together with grafting tapes so that their cambium layers were aligned. The progress of the graft was checked through untying the tapes after two months. After the parts were well united, the top portion of the rootstock immediately above the graft union was cut off followed by the removal of the bottom portion (root system) of the scion below the graft union. The graft union was then completed with the scion being solely dependent on the root system of the rootstock. The graft success was determined as the survival rate four months after the beginning of grafting.

2.2 Preparation of microsomal fraction and measurement of plasma membrane H^+-ATPase activity.

Samples collected for PM H^+-ATPase activity assay included rootstock leaves removed from the intended graft union before grafting, rootstock stems immediately above the graft union two months after grafting, scion apical

leaves after completion of grafting and graft union sections from both failed an. The microsomal fraction were prepared at 4 ^{o}C as previously described (Zhou et al., 2014). Briefly, tissues (leaves or stems at ~50 g) were homogenised in ice-cold medium (1:3 w/v) [0.1 M Tris-Mes pH 7.7 with 2 mM dithiothreitol (DTT), 20 mM ethylene diamine tetraacetic acid (EDTA), 20 mM ethylene glycol bis (2-aminoethyl) tetraacetic acid (EGTA), 0.5 M sucrose, 10 mm ascorbic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2% (w/v) insoluble polyvinylpyrrolidone (PVP)]. The homogenate was filtered through several layers of cheesecloth and centrifuged at 13,000 g for 15 min. The supernatant was then centrifuged for 30 min at 100,000 g to obtain microsomal pellet. The pellets were resuspended in suspension medium [5 mM KPi, pH7.8, 0.33 M sucrose, 2 mM DTT, 3 mM KCl] for immediate H^+-ATPase activity assay.

ATP hydrolytic activity was measured at 38 ^{o}C for 30 min following a previously described procedure (Zhou et al., 2014) with modification. The reaction was initiated by addition of microsomal suspension (~15 µg of microsomal proteins) to a reaction mix [50 mM Tris-MES, pH 6.5, 3 mM Tris-ATP, 50 mM KCl, 3 mM MgSO4, 0.5 mM ammonium molybdate, 50 mM KNO3, 0.1% [w/w] Triton X-100]. The amount of inorganic phosphate released from ATP was determined as described (Kasamo, 1990). The activities of vanadate-sensitive ATPase were calculated form the difference between activities in the absence and presence of 1 mM Na_3VO_4. The activity was presented as µmol Pi/min/mg Protein. Protein concentration was determined according to Bradford (Bradford, 1976).

2.3. Statistical analyses

Significant differences were tested using analysis of variance (ANOVA) followed by Tukey's multiple comparison test at $P<0.05$. The relationship between graft success and stem PM H^+-ATPase activity was determined by linear regression analysis under SPSS software (IBM SPSS Statistics version 24).

3. Results

3.1. Success of breadfruit grafting onto interspecific rootstocks of marang and pedalai

All grafts were un-tied to check the adhesion before removal of the scion's self-roots. At this stage, 100% of the grafts were adhered well for both rootstocks. A successful grafting event was initially identified by survival for over 4 weeks after complete removal of the scion roots, leaving the grafted plant solely dependent on the root system of rootstock (Fig 1C). At 3 to 5 months after grafting, success was confirmed by appearance of shoot

growth from the terminal bud of the grafted scions along with emergence of new utmost leaves (Fig. 1E, Fig. 2), a characteristics of active growth pattern in breadfruit (Zhou and Underhill, 2016). At the end of 4 months (2 months after scion root removal), a survival rate of 25 % was observed for grafted breadfruit on marang (7 out of 28 attempted), and a survival rate of 9 % was for grafted breadfruit on pedalai (3 out of 32 attempted).

3.2. Comparison of PM H+-ATPase activity from graft unions

To investigate the cause of low graft survival, we examined the physical and biochemical properties of the graft unions. All the grafts adhered with similar strength held between grafting partners in the graft junction (fig. 1C), even though the final graft survival was low. These suggest the healing and adhesion may come from a passive wounding response, not necessarily associated with functional vascular connection and graft success (Melnyk, 2017). The majority of the failed grafts showed leaf wilting and drooping within 2 weeks after removal of their scion's own roots. These stress symptoms prompted us to examine the activity of PM H+-ATPase, a primary proton pump responsible for nutrient uptake and turgor regulation (Palmgren, 2001). The graft union of these grafts were sampled for microsomal ATPase activity assay in comparison with those of the healthy (successful) grafts. These showed PM H+-ATPase activities from graft unions of successful grafts were significantly higher than those of unsuccessful grafts for both rootstocks, with 104% and 89% higher for marang and pedalai rootstocks respectively (Fig. 3).

3.3. PM H^+-ATPase activity in rootstock leaves before grafting and graft success

In order to examine whether high PM H+-ATPase activity is required for graft survival, we examined the PM H^+- ATPase activity in rootstocks before grafting. At the beginning of grafting, rootstock leaves peeled from the intended graft section (potential bound section) (Fig. 1A) were sampled for microsomal preparation and ATPase activity assay. These showed the PM H+-ATPase activities of marang leaves were in the range of 0.05 to 0.40 µmol Pi/min/mgProtein, whereas those of pedalai were in the range of 0.07 to 0.37 µmol Pi/min/mgProtein. Rootstocks of each species were grouped according to their levels of PM H^+-ATPase activity, with ≤ 0.14, 0.15 to 0.25 and ≥ 0.26 µmol Pi/min/mgProtein representing the low, medium and high activities respectively. Each species group had at least 7 rootstocks (n ≥7). The graft survival was determined four months after the beginning of grafting (two months after the completion of grafting). These results showed

for both marang and pedalai rootstocks, groups in the low PM H^+-ATPase activities had significantly lower survival rate compared to those in high activity groups, with 10% vs 63% and 0% vs 36% for marang and pedalai rootstocks respectively (Fig. 4). However, those groups between the medium and high activities was not significantly different for both rootstock species (Fig. 4).

3.4. PM H^+-ATPase activity in rootstock stems and graft success

To further examine the relationship of PM H^+-ATPase activity to graft success**,** rootstock stems immediately above the graft union were sampled for microsomal preparation and ATPase activity analysis in order to avoid the destructive analysis of graft union and scion stems (Fig. 1C). These showed, at the end of two months the stem PM H^+-ATPase activities of marang rootstocks were in the range of 0.031 to 0.19 µmol Pi/min/mgProtein, whereas those of pedalai were in the range of 0.028 to 0.17 µmol Pi/min/mgProtein. Rootstocks of the same species were also grouped according to their levels of stem ATPase activity, with ≤ 0.05, 0.06 to 0.10 and ≥ 0. 11 µmol Pi/min/mgProtein being low, medium and high activities respectively. Each species group had at least 9 rootstocks ($n \geq 9$). These showed the graft success of breadfruit onto marang or pedalai significantly increased as the rootstock stem ATP activities increased from low, medium to high levels (Fig. 5). Linear regression analysis (Fig. 5) confirmed there was a significant, positive relationship between the PM H^+-ATPase activities of microsomes prepared from the rootstock stems at this stage and the graft success for both marang ($r(7)= 0.9203$; $P= 0.00043$) and pedalai rootstocks ($r(7) = 0.8820$, $P= 0.0017$).

3.5. Changes of PM-H^+-ATPase activity in scion leaves after completion of grafting.

After completion of grafting, the scion apical leaves grafted on marang rootstocks were assayed for microsomal PM H^+-ATPase activity every two days for 6 days, with day 0 as the time immediately before removal of the scion's own roots two months after grafting. The enzyme activities of the survived scions were compared with those unsuccessful grafts four months after grafting. These results showed there were distinct difference in the pattern of PM H^+-ATPase activity change between the two groups (Fig. 6). At day 2, the PM H^+-ATPase activities significantly decreased after removal of the root system of the scions regardless of the final graft outcome, with over 35% reduction compared to day 0. In the unsuccessful grafts, the enzyme activities continued to drop, with an 84% decrease at day 6 compared to those at day 0. In contrast, in the survived

scions, the PM H^+-ATPase activity gradually recovered after day 2, with activity levels at day 6 being relatively close to those at day 0 (Fig. 6). As a result, at day 6, the scion PM H^+-ATPase activities of the successfully grafted group were 4-fold higher than those of the unsuccessful grafts (Fig. 6).

4. Discussion

In this study, breadfruit (*Artocarpus altilis)* was successfully grafted onto interspecific rootstocks, marang (A. odoratissimus) and pedelai (A. sericicarpus) (Fig. 1 & 2). The graft success of 25% for marang and 9% for pedalai species, are much lower compared to previous observation on breadfruit grafting to species, *A. camansi* and *A. mariannensis* (Nandwani and Kuniyuki, 2005; Medagoda and Chandrarathna, 2007). The results may reflect the phylogenetic relationship between breadfruit and these *Artocarpus* species. *A. camansi* and *A. mariannensis* are the closest relatives of breadfruit species, with the three species together forming a separate clade previously treated as one species, *A. communis,* whereas pedalai (*A. sericicarpus*) and marang (*A. doratissimus*) are represented in a separated section and series respectively from breadfruit species although they are all under the subgenus *Artocarpus (Zerega et al., 2010)* . In order to identify factors causing the low graft success rate, we examined the graft union for physical and biochemical difference between the survival and the failed grafts. We found that while 100% of graft union adhered well between grafting partners, with similar strength in graft junction (Fig. 1), the graft union of successful grafts showed significantly higher PM H^+-ATPase activity compared to those of unsuccessful grafts (Fig. 3). PM H^+-ATPase is an integral membrane proton pump that plays key roles in nutrient acquisition and partitioning (Palmgren, 2001). The enzyme generates the proton-motive force that drives the uptake of nutrients such as sugars and ions across the plasma membrane of growing cells (Michelet and Boutry, 1995; Palmgren, 2001). Our results are in agreement with the fundamental role of PM H^+-ATPase in plant growth and physiological responses, including cell expansion, phloem loading and stress adaptation (Fromm et al., 1989; Michelet and Boutry, 1995; Palmgren, 2001). Expression of PM ATPase protein was differentially upregulated in the graft union 30 days after grafting of pecan (*Carya illinoensis*) (Mo et al., 2017), together with upregulation of PM ATPase genes at 8d, 15d and 30 day after grafting (Mo et al., 2018). Upregulation of both PM H^+-ATPase protein and genes was observed in the graft union of Hickory (*Carya cathayensis*) (Qiu et al., 2016; Xu et al., 2017). Members of PM ATPase gene family were upregulated at the graft interface of litchi (*Litchi chinensis*) for compatible graft at 14 d and 21 d after grafting (Chen et al., 2017). PM ATPase genes were also upregulated at the graft union of grapevine

heterograft 14 day after grafting (Cookson et al., 2014). Furthermore, at least seven isoforms of PM ATPase genes, including *At1g80660*, *At3g47950*, *At5g57350*, *At4g29900, At1g17260*, *At2g18960* and *At3g42640* were recently reported being differentially up-regulated either asymmetrically or symmetrically around the graft union of *Arabidopsis thaliana* from 6 h to 240 h after grafting (Melnyk et al., 2018). These evidences together suggest PM H^+-ATPase may be required for the successful development of graft union.

We further investigated whether high level of PM H^+-ATPase activity before grafting could be beneficial for graft survival. This let to our findings that rootstocks with lowest PM H^+-ATPase activity in leaf microsomes before grafting had significantly lower graft survival than those with highest enzyme activity (Fig. 4). Through selection of rootstocks with high levels of PM H^+-ATPase activity from rootstock leaves before grafting, the graft success of breadfruit could increase to 60% for marang and 30% for pedalai rootstocks (Fig. 4). Furthermore, there was a positive correlation ($P < 0.01$) between graft success and the PM H^+-ATPase activities measured from the rootstock stem microsomes two months after grafting (Fig. 5). These results suggest PM H^+-ATPase may have a role in graft union formation. As a primary ion pump, PM H^+-ATPase is thought to affect plant growth and development through two basic processes: 1) energization of nutrient uptake across plasma membrane; and 2) stimulation of cell expansion through apoplast acidification (Stahlberg and Van Volkenburgh, 1999; Ehlert et al., 2011; Spartz et al., 2014). Higher expression of PM H^+-ATPase has been reported for those tissues where high rates of solute exchange across plasma membrane is required, such as guard cells, phloem and xylem parenchyma cells (Michelet and Boutry, 1995; Palmgren, 2001; Arend et al., 2002). Many evidences have indicated that graft union is highly active in metabolic flux with significant upregulation of sucrose and energy metabolism during graft union formation (Melnyk and Meyerowitz, 2015; Muneer et al., 2015; Mo et al., 2017; Xu et al., 2017). In fact, application of sucrose into growth medium or soils before grafting was found to improve graft success (Marsch-Martinez et al., 2013; Sanabam et al., 2015; Dabirian and Miles, 2017). The uptake of most nutrients into cambial cells is an energy-dependent process under the control of PM H^+-ATPase (Michelet and Boutry, 1995; Palmgren, 2001). Therefore, a very low activity of PM H+-ATPase in graft union or rootstocks (Fig. 3-5) may suggest there is not enough proton-motive force to sustain the uptake of nutrients essential for growth of graft union cells. Furthermore, PM H^+-ATPase has long been recognised for promoting cambial cell differentiation through turgor-driven cell expansion (Palmgren, 2001). Cambial cell differentiation leading to vascular regeneration is considered as a hallmark of graft success (Pina and Errea, 2005; Yin et al., 2012; Melnyk, 2017). Strong evidences have demonstrated the operation of proton pump in uptake of K^+ and other nutrients essential for osmotic regulation and cell enlargement in differentiating cambial cells (de Boer et

al., 1985; Pavlovkin et al., 2002; Arend et al., 2004). Two plasma membrane H^+-ATPases were upregulated in wood-forming tissues of maritime pine (*Pinus pinaster*) during the development of secondary xylem (Paiva et al., 2008). Induced differentiation of vascular bundles and functional connection in *Ricinus communis* were associated with high PM H^+-ATPase activity in the xylem and phloem parenchyma cells (Pavlovkin et al., 2002). PM H^+-ATPase is also a key enzyme for H^+ efflux resulting in cell-wall loosening and cell extension in differentiating tissues (Arend et al., 2002; Fromm, 2013; Spartz et al., 2014). Reduced PM H^+-ATPase activity was found to be associated with low cell extensibility in aging organs (Sveinsdottira et al., 2009). In this context, a very low PM H^+-ATPase activity in rootstocks may suggest a low capacity in cell expansion of grafting tissues. Notably, high capacity to divide and differentiate is one of the important criteria for rootstock selection in order to achieve high graft success in horticulture practices (Melnyk and Meyerowitz, 2015; Melnyk, 2017).

The induction of PM H^+-ATPase by auxin is well documented during cambial growth (Palmgren, 2001; Pavlovkin et al., 2002; Spartz et al., 2014). Accumulating evidences have also demonstrated that auxin response play an important role in the differentiation of vascular tissues during graft union formation (Arend et al., 2002; Aloni et al., 2010; Melnyk, 2017). It is not known whether the correlation of PM H^+-ATPase activity to graft success in our current study is associated with auxin response. In addition, the possibility of mRNA transfer through graft union or exchange of genetic materials between rootstock and scion cannot be ruled out (Stegemann and Bock, 2009; Xia et al., 2018). Further investigation into the nature of the PM H^+-ATPase increase and its role in graft union formation may provide opportunity for graft improvement through PM H^+-ATPase monitoring and manipulation.

The results that levels of PM H^+-ATPase activity in scion leaves of grafted plants decreased immediately after removal of the scion roots (Fig. 6) suggests a significant shock may occur as a result of separation. The results are consistent with the function of PM H^+-ATPase in triggering primary response to abiotic stresses including water-deficiency, osmotic and nutrients stress, and reflect early disturbance in ion homeostasis and metabolism (Portillo, 2000; Zhou et al., 2014). In particular, PM H^+-ATPase play a crucial role in stomatal closure (Elmore and Coaker, 2011), an important stress response for control of water loss that is critical for graft survival during the early stage of graft union establishment. In fact, reduction in transpiration through application of stomata-closing agents has been shown to improve graft success (Dabirian and Miles, 2017). Therefore, the early recovery of PM H^+-ATPase activity in grafted plants may not only reflect the return of electrochemical gradient

to sustain nutrient uptakes and cellular homeostasis, but also suggest the restoration of capability to control water loss, leading to a higher chance of survival.

Taken together, based on the findings that higher level of PM H^+-ATPase activity was found in the graft union of breadfruit scions successfully grafted onto interspecific rootstocks of marang and pedalai, we found a positive relationship between PM H+-ATPase activity and graft success. First, rootstocks with very low PM H+-ATPase activity in leaves before grafting had significantly lower graft survival than those with high enzyme activity. Second, there was a positive correlation between graft success and the PM H+-ATPase activity measured from the rootstock stems two months after grafting. Third, the recovery of PM H^+-ATPase activity from scion leaves was associated with the long-term survival of the grafted plants. The information may provide opportunity to develop screening strategy for rootstock selection to improve graft success for breadfruit dwarfing.

5. Acknowledgments

This work was funded by the Australia Centre for International Agriculture Research (ACIAR) through project HORT 2014/077. The authors are indebted to the reviewers whose comments have improved the manuscript.

6. References

Aloni, B., Cohen, R., Karni, L., Aktas, H. and Edelstein, M. (2010) Hormonal signaling in rootstock-scion interactions. Scientia Horticulturae, **127**, 119–126.

Arend, M., Monshausen, G., Wind, C., Weisenseel, M. H. and Fromm, J. (2004) Effect of potassium deficiency on the plasma membrane H(+)-ATPase of the wood ray parenchyma in poplar. Plant, Cell & Environment **27**, 1288-1296.

Arend, M., Weisenseel, M. H., Brummer, M., Osswald, W. and Fromm, J. H. (2002) Seasonal changes of plasma membrane H(+)-ATPase and endogenous ion current during cambial growth in poplar plants. Plant Physiology, **129**, 1651-1663.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitative microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, **72**, 248-252.

Chen, Z., Zhao, J. T., Hu, F. C., Qin, Y. H., Wang, X. H. and Hu, G. B. (2017) Transcriptome changes between compatible and incompatible graft combination of Litchi chinensis by digital gene expression profile. Scientific Reports, **7**, 3954-3954.

Cookson, S. J., Moreno, M. J. C., Hevin, C., Mendome, L. Z. N., Delrot, S., Magnin, N., Trossat-Magnin, C. and Ollat, N. (2014) Heterografting with nonself rootstocks induces genes involved in stress responses at the graft interface when compared with autografted controls. Journal of Experimental Botany, **65**, 2473–2481.

Dabirian, S. and Miles, C. A. (2017) Antitranspirant Application Increases Grafting Success of Watermelon. HortTechnology **27**, 494-501.

Dabirian, S. and Miles, C. A. (2017) Increasing Survival of Splice-grafted Watermelon Seedlings Using a Sucrose Application. Hortscience, **52**, 579-583.

Daley, O., Gloster, M. and Roberts-Nkrumah, L. B. (2012) Assessment and characterization of damage by hurricane Tomas to major tree crops with special emphasis on breadfruit (*artocarpus altilis*) and breadnut (*artocarpus camansi*) in St. Lucia and St. Vincent and the Grenadines. Proceedings of the Caribbean Food Crop Society, **48**, 124-131.

de Boer, A. H., Katou, K., Mizuno, A., Kojima, H. and Okamoto, H. (1985) The role of electrogenic xylem pumps in K+ absorption from the zylem of Vigna unguiculata: the effects of auxin and fusicoccin. Plant, Cell & Environment, **8**, 579–586.

Ehlert, c., Plassard, c., Cookson, s. j., Tardieu, f. and Simonneau, t. (2011) Do pH changes in the leaf apoplast contribute to rapid inhibition of leaf elongation rate by water stress? Comparison of stress responses induced by polyethylene glycol and down-regulation of root hydraulic conductivity. Plant, Cell & Environment, **34**, 1258–1266.

Elmore, J. and Coaker, G. (2011) The Role of the Plasma Membrane H+-ATPase in Plant–Microbe Interactions. Molecular Plant, **4**, 416-427.

Fromm, H., Katagiri, F. and Chua, N. (1989) An octopine synthase enhancer element directs tissue specific expression and binds ASF-1, a factor from tobacco nuclear extracts. Plant Cell, **1**, 977-984.

Fromm, J. (2013) Xylem development in trees: from cambial divisions to mature wood cells. in. Fromm, J. (Eds). Cellular Aspects of Wood Formation. Plant Cell Monographs. 20, Springer. Berlin, Heidelberg

Kasamo, K. (1990) Mechanism for the activation of plasma membrane H+-ATPase from rice (*Oryza sativa* L.) culture cells by molecular species of a phospholipid. Plant Physiology, **93**, 1049-1052.

Marsch-Martinez, N., Franken, J., Gonzalez-Aguilera, K. L., de Folter, S., Angenent, G. and Alvarez-Buylla, E. R. (2013) An efficient flat-surface collar-free grafting method for Arabidopsis thaliana seedlings. Plant Methods, **9**, 14.

Medagoda, I. and Chandrarathna, K. W. M. C. J. (2007) Grafting of breadfruit (artocarpus altilis) using breadnut *(Artocarpus camansi*) as root stock. Acta Horticulturae, **757**, 149-152.

Melnyk, C. W. (2017) Plant grafting: insights into tissue regeneration. Regeneration, **4**, 3–14.

Melnyk, C. W., Gabel, A., Hardcastle, T. J., Robinson, S., Miyashima, S., Grosse, I. and Meyerowitz, E. M. (2018) Transcriptome dynamics at Arabidopsis graft junctions reveal an intertissue recognition mechanism that activates vascular regeneration. Proceedings of the National Academy of Sciences of the United States of America, **115**, E2447–E2456.

Melnyk, C. W. and Meyerowitz, a. E. M. (2015) Plant grafting. Current Biology, **25**, R183-R188.

Michelet, B. and Boutry, M. (1995) The plasma membrane H+-ATPase (A highly regulated enzyme with multiple physiological functions). Plant Physiology, **108**, 1-6.

Mo, Z., Feng, G., Su, W., Liu, Z. and Peng, F. (2018) Transcriptomic Analysis Provides Insights into Grafting Union Development in Pecan (Carya illinoinensis). Genes, **9**, 71.

Mo, Z. H., He, H. Y., Su, W. C. and Peng, F. R. (2017) Analysis of differentially accumulated proteins associated with graft union formation in pecan (Carya illinoensis). Scientia Horticulturae, **224**, 126-134.

Muneer, S., Ko, C. H., Soundararajan, P., Manivnnan, A., Park, Y. G. and Jeong, B. R. (2015) Proteomic Study Related to Vascular Connections in Watermelon Scions Grafted onto Bottle-Gourd Rootstock under Different Light Intensities. Plos One, **10**, e0120899.

Nandwani, D. and Kuniyuki, A. H. (2005) Grafting and improvement of breadfruit production in micronesia. Proceedings of the International Symposium on Harnessing the Potential of Horticulture in the Asian-Pacific Region. Acta Horticulturae, **694**, 307-310.

Paiva, J. A. P., Garnier-Géré, P. H., Rodrigues, J. C., Alves, A., Santos, S., Graça, J., Provost, G. L., Chaumeil, P., Silva-Perez, D. D., Bosc, A., Fevereiro, P. and Plomion, C. (2008) Plasticity of maritime pine (Pinus pinaster) wood-forming tissues during a growing season. New Phytologist, **179**, 1080-1094.

Palmgren, M. G. (2001) Plant plasma membrane H+-ATPase: Powerhouses for nutrient uptake. Annual Review of Plant Physiology and Plant Molecular Biology, **52**, 817–845.

Pavlovkin, J., Okamoto, H., Wächter, R., Läuchli, A. and Ullrich, C. (2002) Evidence for high activity of xylem parenchyma and ray cells in the interface of host stem and Agrobacterium tumefaciens-induced tumours of *Ricinus communis*. Journal of Experimental Botany, **53**, 1143-1154.

Pina, A. and Errea, P. (2005) A review of new advances in mechanism of graft compatibility–incompatibility. Scientia Horticulturae, **106**, 1-11.

Portillo, F. (2000) Regulation of plasma membrane H^+-ATPase in fungi and plants. Biochimica et Biophysica Acta, **1469**, 31-42.

Qiu, L., Jiang, B., Fang, J., Shen, Y., Fang, Z., Rm, S., Yi, K., Shen, C., Yan, D. and Zheng, B. (2016) Analysis of transcriptome in hickory (Carya cathayensis), and uncover the dynamics in the hormonal signaling pathway during graft process. BMC Genomics, **17**, 935.

Ragone, D. (1997) Breadfruit, *Artocarpus altilis* (Parkinson) Fosberg - Promoting the conservation and use of underutilized and neglected crops. 10, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, (Germany). http://www.jeffmarck.net/breadfruitdocs/Ragone_1997_IPGRI_breadfruit.pdf

Ragone, D. (2006) *Artocarpus altilis* (breadfruit). in. Elevitch., C. R. (Eds). Traditional trees of Pacific Islands: their culture environment and use. Permanent Agriculture Resources Holualoa, Hawaii**,** pp 85-100

Roberts-Nkrumah, L. B. (2012) Breadnut and breadfruit propagation, a manual for commercial propagation, Department of Agriculture, Food and Agriculture Organization of the United Nations. ttp://www.fao.org/3/a-i3085e.pdf

Sanabam, R., Singh, N. S., Handique, P. J. and Devi, H. S. (2015) Disease-free khasi mandarin (Citrus reticulata Blanco) production using in vitro microshoot tip grafting and its assessment using DAS-ELISA and RT-PCR. Scientia Horticulturae, **189**, 208-213.

Solomon, F. J. and Roberts-Nkrumah, L. B. (2008) An evaluation of factors influencing successful grafting of breadfruit on chataigne rootstock. 2008 Proceedings of the Caribbean Food Crops Society, **44**, 304-312.

Spartz, A. K., Ren, H., Park, M. Y., Grandt, K. N., Lee, S. H., Murphy, A. S., Sussman, M. R., Overvoorde, P. J. and Gray, W. M. (2014) SAUR inhibition of PP2C-D phosphatases activates plasma membrane H+-ATPases to promote cell expansion in *Arabidopsis*. Plant Cell, **26**, 2129-2124.

Stahlberg, R. and Van Volkenburgh, E. (1999) The effect of light on membrane potential, apoplastic pH and cell expansion in leaves of Pisum sativum L. var. Argenteum. - Role of the plasma-membrane H+-ATPase and photosynthesis. Planta, **208**, 188-195.

Stegemann, S. and Bock, R. (2009) Exchange of genetic material between cells in plant tissue grafts. Science, **324**, 649–651.

Sveinsdottira, H., Yan, F., Zhu, Y. Y., Peiter-Volk, T. and Schubert, S. (2009) Seed ageing-induced inhibition of germination and post-germination root growth is related to lower activity of plasma membrane H+-ATPase in maize roots. Journal of Plant Physiology, **166**, 128-135.

Xia, C., Zheng, Y., Huang, J., Zhou, X., Li, R., Zha, M., Wang, S., Huang, Z., Lan, H., Turgeon, R., Fei, Z. and Zhang, C. (2018) Elucidation of the Mechanisms of Long-Distance mRNA Movement in a Nicotiana benthamiana/Tomato Heterograft System. Plant Physiology **177**, 745-758.

Xu, D., Yuan, H., Tong, Y., Zhao, L., Qiu, L., Guo, W., Shen, C., Liu, H., Yan, D. and Zheng, B. (2017) Comparative Proteomic Analysis of the Graft Unions in Hickory (Carya cathayensis) Provides Insights into Response Mechanisms to Grafting Process. Frontiers in Plant Science, **8**, 676.

Yin, H., Yan, B., Sun, J., Jia, P., Zhang, Z., Yan, X., Chai, J., Ren, Z., Zheng, G. and Liu, H. (2012) Graft-union development: a delicate process that involves cell–cell communication between scion and stock for local auxin accumulation. Journal of Experimental Botany, **63**, 4219–4232.

Zerega, N., Ragone, D. and Motley, T. J. (2004) Complex origins of breadfruit (Artocarpus altilis, Moraceae): Implications for human migrations in Oceania. American Journal of Botany, **91**, 760-766.

Zerega, N., Supardi, M. N. N. and Motley, T. J. (2010) Phylogeny and recircumscription of Artocarpeae (Moraceae) with a focus on Artocarpus. Systematic Botany, **35**, 766-782.

Zhou, Y., Pan, X., Qu, H. and Underhill, S. J. R. (2014) Low temperature alters plasma membrane lipid composition and ATPase activity of pineapple fruit during blackheart development. Journal of Bioenergetics and Biomembranes, **46**, 59-69.

Zhou, Y., Taylor, M. B. and Underhill, S. J. R. (2014) Dwarfing of breadfruit (*Artocarpus altilis*) trees: opportunities and challenges. American Journal of Experimental Agriculture, **4**, 1743-1763

Zhou, Y. and Underhill, S. (2016) Breadfruit (Artocarpus altilis) gibberellin 2-oxidase genes in stem elongation and abiotic stress response. Plant Physiology and Biochemistry, **98**, 81-88.

Figure legend

Fig. 1 Representatives of breadfruit approach grafting onto interspecific rootstocks. A. breadfruit scion plant (right) and a rootstock seedling (left) were brought next to each other. Leaves and a slice of the stem (3 ~ 5 cm long and 40% thickness) were removed at the sections where the graft union was to occur (red arrows). B. Alignment of two peeled surfaces with grafting tapes. C. Completion of grafting two months later. After two parterns were well united (see inserted picture), the top portion of the rootstock above, and the bottom portion of the scion below the graft union were cut away (red arrows), with the scion being solely dependent on the root system of the rootstock. D-E, Grafted breadfruit plants on pedalai (D) and marang (E) rootstocks 3 months after grafting. F-G, Grafted breadfruit plants on pedalai (F) and marang (G) rootstocks 6 months after grafting. Sampes collected for plasma membrane H^+-ATPase assay: S1, rootstock leaves from the potential graft union; S2, stems from the removed rootstock portions; S3, graft union; S4, apical scion leaves collected at 2-day intervals after the completion of grafting.

Fig. 2 Elongation of scion stems in grafted breadfruit plants. The elongation of scion stems was measured above the graft union from 3 months after grafting (equivalent to 1 month after removal of the scion own roots, see Fig. 1C). All values represent mean ± SE of three biological replicates.

Fig. 3 Comparison of plasma membrane H^+-ATPase activities from graft union of successful and unsuccessful grafts on to marang and pedalai rootstocks. ATP hydrolytic activity was assayed in the microsomal fractions prepared from the graft union two weeks after grafting completion (after removal of scion's own roots, Fig. 1C, S4). PM H-ATPase activity, represented as the vanadate-sensitive ATPase, is presented as µmol Pi/min/mg Protein. All values represent mean ± SE of four biological replicates. Values with different letter are significantly different (P<0.05).

Fig. 4 Relationship between plasma membrane H^+-ATPase activities from rootstock leaves and graft success of breadfruit onto marang and pedalai rootstocks. ATP hydrolytic activity was assayed in the microsomal fractions prepared from rootstock leaves of the intended graft union before grafting (Fig. 1A). The activity of PM H-ATPase, represented as the vanadate-sensitive ATPase, was calculated from the difference between activities in the absence and presence of 1 mM Na_3VO_4. Rootstocks of each species were grouped according to the levels of

PM H^+-ATPase activity in the microsomal fractions, with ≤ 0.14, 0.15 to 0.25 and ≥ 0.26 µmol Pi/min/mgProtein representing the low, medium and high activity groups respectively. Each group contained at least seven rootstocks for each experiment. The graft success was determined four months after grafting. All values represent mean ± SE of three separate experiments. Values with different letter are significantly different (P<0.05).

Fig. 5 Relationship between plasma membrane H^+-ATPase activities from rootstock stems and graft success of breadfruit onto marang and pedalai rootstocks. A. Rootstock PM H^+ATPase activity grouping and graft success. ATP hydrolytic activity was assayed in the microsomal fractions prepared from rootstock stems immediately above the graft union two months after grafting (Fig. 1C). The activity of PM H-ATPase, represented as the vanadate-sensitive ATPase, was calculated from the difference between activities in the absence and presence of 1 mM Na_3VO_4. Rootstocks of each species were grouped according to PM H^+-ATPase activity levels in the microsomal fraction, with ≤ 0.05, 0.06 to 0.10 and ≥ 0. 11 µmol Pi/min/mgProtein for low, medium and high activity groups respectively. Each group had at least nine rootstocks for each experiment. All values represent mean ± SE of three separate experiments. Values with different letter are significantly different (P<0.05). B. Linear regression for relationship between rootstock stem PM H^+ATPase activities and graft success. Correlation coefficient: r(7)= 0.9203; P = 0.00043 for marang; r(7) = 0.8820, P= 0.0017 for pedalai. Activities of PM H^+ATPase are presented as µmol Pi/min/mg Protein. All activity values represent mean of at least nine samples. The graft success is based on the percentage of survival four months after grafting.

Fig. 6 Changes in plasma membrane H^+-ATPase activity of scion leaves after removal of scion roots. ATP hydrolytic activity was assayed in the microsomal fractions prepared from apical leaves of scions before (day 0) and after (Day 2 to Day 6) the removal of scion roots (Fig. 1C). The activities of PM H-ATPase, represented as the vanadate-sensitive ATPase, are presented as µmol Pi/min/mg Protein. The graft outcome, as successful or unsuccessful were determine four months after grafting. The values represent mean ± SE of three separate experiments and are expressed as percentage of the activity at day 0 (100%). Values with different letter are significantly different (P<0.05).