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Unparalleled *Agrobacterium* gene transfer to cells near elongation zone of emerging monocot leaves

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Abstract: In the early days of plant transformation, monocot plant species were thought to be recalcitrant to *Agrobacterium* infection. But now it is routine to produce monocot transgenic plants in different species after *Agrobacterium* mediated gene transfer to the immature zygotic embryos or somatic embryos. However, these known *Agrobacterium* infectable monocot tissues are tiny with the infection limited to surface cell layers and most often happening under narrowly defined conditions. Here we report, a curious phenomenon of *Agrobacterium* infecting almost all cells spread across an astonishing ~30cm^2 area composed of an estimated 75 million confluent cells, at the base of emerging leaves in corn. This highly transformable region extends from the upper epidermis to the lower epidermis, through all the inner cell layers including the cells within the bundle sheath, making the zone after transformation, indistinguishable from the tissue derived from any transgenic plant. Evidences suggest the chromosomal integration of the transgene in these transformed cells. Surprisingly, neither pre *vir* induction nor wounding is needed for this extensive infection of monocot cells by *Agrobacterium*. Further, other organs which are known homologs of leaf, like the husk leaves, the glumes of spikelets and scutellum of embryos are also extensively infectable in their early stage of development while the infection of roots was rare. The extensive competency for *Agrobacterium* infection of the emerging leaf base tissue, which in nature

is concealed deep inside the multiple whorls of older leaf sheaths, appeared to be a common occurrence in monocot plant species. Evidences suggest, the limitation for *Agrobacterium* infection of monocot leaves are structural barriers acquired during cell maturation and probably has less to do with the nature of their wound response.

Key words: *Agrobacterium*, corn, sugarcane, wheat, monocot, leaf, transformation, elongation zone

Abbreviations: IZ – infectable zone; GUS - β-glucuronidase gene; GFP - green fluorescent protein;

Introduction:

In the early years of plant transformation, monocots were thought to be outside the host range of *Agrobacterium* (Potrykus, 1990). This is in contrast to the dicot plants, many species of which were known to be natural hosts of *Agrobacterium*. Many factors had been attributed for this observed lack of efficient gene transfer to monocot plants, the important ones being linked to their wound response. The dicot plants' cells upon wounding secrete *vir* gene inducing phenolic compounds such as acetosyringone. In addition wounding in dicots induces active cell division at the injury site and both of these wound responses are thought to be important for *Agrobacterium* infection. The observed recalcitrance of monocot plants to *Agrobacterium* infection is widely believed to be because of their deficiency in the above two wound responses *viz*., the activation of cell division and the secretion of phenolic compounds (Li et al., 2000).

The first evidence of *Agrobacterium* mediated gene transfer to cereals was obtained through Agroinfection, a method of transferring a *vir*al genome cloned within the T-DNA, which when successfully transferred by the *Agrobacterium* to the host plant would multiply as viral particles and spread systemically throughout the plant to produce the *vir*al symptoms (Grimsley, 1987). Direct proof of *Agrobacterium* gene transfer to cereals was shown when the first transgenic rice plants were produced, where the transfer of gene was mediated through *Agrobacterium* (Hiei et al., 1994). This was soon followed

by reports of transformation of other economically important cereals (Ishida et al., 1996; reviewed in Ji et al., 2013) involving similar method of gene transfer. It is routine to produce transgenic monocot plants after *Agrobacterium* mediated gene transfer (reviewed by Hiei, 2014). This later success with transformation of monocot plants using *Agrobacterium* for gene delivery has been attributed to crossing the many technical difficulties around the critical factors and the narrow optimal windows of these critical factors that affect monocot transformation (Hiei et al., 2014). These factors mainly are genotype, condition of explants donor plant, tissue culture methods and culture media, pre-treatments of explants, strains of *Agrobacterium*, inducers of *vir*ulence genes, type of plasmid vectors, selection marker genes and selective agents (Hiei et al., 2014).

In spite of this later progress in monocot transformation, most often the *Agrobacterium* transformable tissue employed for making transgenic plants is limited to the scutellum tissue of immature embryos or the somatic embryogenic calli (reviewed by Hiei, 2014; Nannas and Dawe, 2015). Further, the successful transformation of these tissues happens under very narrowly defined conditions and is restricted to the surface cell layers. Even with the Agroinfection method, gene transfer was possible only when transformation was attempted near the meristamatic zones and the Agroinfection could not be achieved when leaf tissues was employed (Grimsley et al., 1999). Though there are now recent reports of development of transient methods of gene expression in leaves of rice (Andrieu et al., 2012) and switch grass (VanderGheynst et al., 2008), the organs / tissue types and the number of cells amenable for transformation by *Agrobacterium* are extremely small in monocot plant species. This is in contrast to the dicot plant species, where the transgenic plants can be produced from multitude of explants like leaf pieces, cotyledons, stem segments, callus suspension cultures and germinating seeds (Hansen and Wright, 1999) and roots (Valvekens et al., 1988). The relative ease of transformation of dicots through *Agrobacterium* is also reflected in the development of robust transient expression methods like leaf infiltration in many species (Chen et a., 2013, Wroblewski et al., 2005). Such simple methods with comparable effectiveness for transient expression are lacking in monocot plants because of the recalcitrance of most of their tissues for *Agrobacterium* infection.

Here we document *Agrobacterium* mediated gene transfer *to* monocotyledonous plant leaves and their modifications, the extent of which is not reported previously either in monocots or dicot plants. The susceptible period for *Agrobacterium* infection of these organs corresponds to their development period when they are physically hidden and protected by other mature organs. Surprisingly, this extensive *Agrobacterium* infection of monocot tissue does not require wounding or pre-induction of *vir* genes. The infection could be performed *in vitro* or *in planta* and evidences indicate integration of the transgene in the genome of the transformed tissue.

Materials and methods

***Agrobacterium* preparation:**

Agrobacterium (strains LBA4404, ABI, AB30, AB31 and AB32) mobilized with the desired construct (Fig. 1) were grown overnight at 28˚C in an incubator-shaker in LB medium supplemented with kanamycin (40µg/ml) and spectinomycin (31µg/ml). The ABI strain was employed in all experiments except where indicated.

Fig.1 : Schematic representation of constructs used in the experiments (a) GUS construct (b) GFP construct (c) Enhancer trap construct with only a minimal promoter (note: if the left border gets inserted next to an enhancer in the corn genome, GUS gene would become functional). RB: right border, LB: left border, P: full length promoter; mp: minimum promoter, 3': 3' UTR

Co-cultivation medium:

The co-cultivation medium consisted of modified MS basal medium with MS major salts, minor salts and vitamins (Murashige and Skoog, 1962) supplemented with 2,4-D (3mg l^{-1}), sucrose (20g l^{-1}), glucose (10g l^{-1}), proline (0.7g l^{-1}), silver nitrate (3.4mg l^{-1}) and MES (0.5g l^{-1}). The pH was adjusted to 5.4 and solidified with agarose (8g l^{-1}). The ingredients glucose and silver nitrate were added post autoclave, mixed well before dispensing at the rate of 30ml / petriplate (90 X 20mm).

Explant preparation and *Agrobacterium* infection:

Zea mays plants (Monsanto proprietary genotypes ML065, ML086, ML648-11, ML197, LH244, DIDA, Hi-Shell, 900M Gold) were grown up to V6-V7 stage in the greenhouse and shoots were harvested as described in Fig. 2a. The expanded leaf blades i.e. of leaves 1-6 are clipped and the remaining cylindrical shoot (Fig.2b,~ 40cms in length) was further processed. From the cylindrical shoots (Fig.2b), the outer dark green leaf-sheaths of the mature leaves were carefully peeled till the base of the emerging leaf was exposed (Figure 2c). The bottom 0- 9cms zone of the elongating leaf base was cut (Fig.2c) un-furled and was dipped in 0.05% Silwet L-77 for 3mins. Silwet L-77 was then drained out and the leaf was dipped into the desired *Agrobacterium* culture for 6mins. Following this, the leaf was spread on co-cultivation medium and co-cultured at 23˚C for 3-4 days and used for transgene expression analysis.

The other explants used *viz*., tassel (approximately 1-2 weeks before emergence), female flowers and husk leaves (when the cob was in elongation phase), stem (in the elongation phase), immature embryos from 11dap cobs and root tissues were also similarly treated with Silwet L-77 before *Agrobacterium* infection.

Sterilization of explants is not needed for green house grown explants donor plants. For field grown donor plants, the trimmed leaf whorls are dipped in 1% Bavistin for 1hr and further sterilized using 2% hypochlorite for 20 minutes and washed extensively with sterile water.

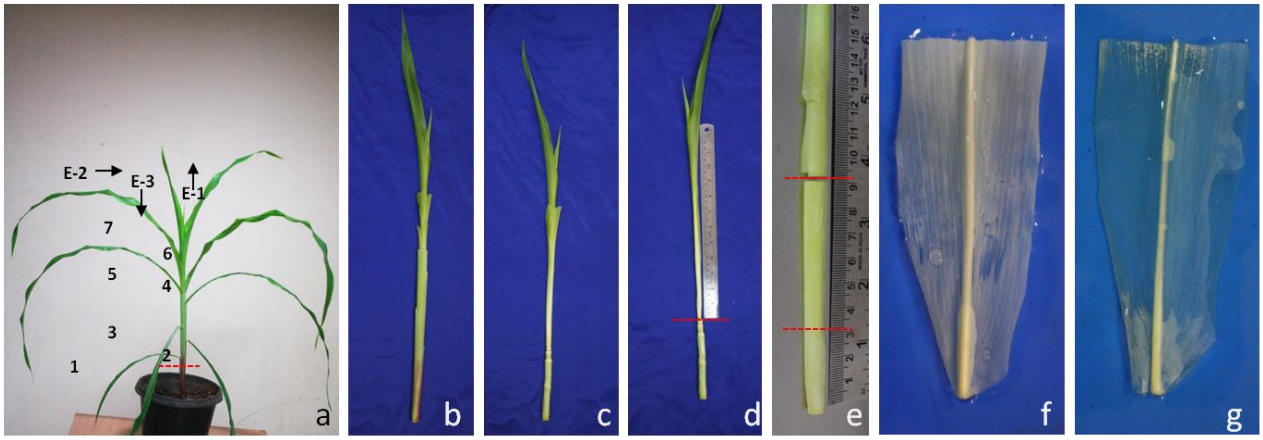

Figure 2: Leaf explant isolation and infection. (a) Shoot of corn plant at V6-V7 stage is cut just above soil level. (Approximate position in V6 plant is indicated with an arrow).
(b) A harvested leaf whorl from which the leaf blades have been removed.
(c) The outer mature leaf bases are peeled off till the base of the emerging leaf (Fig 1a) is exposed.
(d) The leaf whorls are separated from the stem apex through a cut at the stem apex
(e) Aligning against a scale, the region between 3cm to 9cm from the bottom is excised and processed further.
(f) The 3-9cms whorls are gently opened to recover the leaf explants and treated with 0.05 % Silwet L-77 for 3mins
(g) The Silwet treated explants are dipped in *Agrobacterium* culture and kept for 6 mins.
Note: The elongating zone (zone of infection) is in the region between 3 and 9cms from the shoot apex. Usually bases from 2 – 3 emerging leaves would be available for infection from a V6 stage corn plant.

Detergents: Breakthrough, Ampholac YSH, Ethylan NS500, C6330, Ethylan1008, Phospholan PNP, AG6210, Phospholan PE65

***In planta Agrobacterium* infection:**

Though *In planta Agrobacterium* infection could be practiced at any stage of corn growth, best results were observed with V5-V6 stage plants when the 7th or 8th leaves are emerging as they have larger infectable zones. The position of the stem-apex within the leaf whorls is recognized through gentle pressing and feeling of the shoot like structure formed by the whorled leaf bases. The leaf whorls would feel hard towards the bottom where the stem is present and soft towards the tip where the stem is absent within the whorl. The boundary between these regions forms the position of the shoot apex. Typically, at V5-V6 stage, the shoot apex would be near the position of the ligule of the second leaf at the bottom of the plant. The needle of a 1ml syringe carrying about 0.5 - 1ml of *Agrobacterium* suspension is inserted halfway through the leaf whorls (usually 0.5-1cm depth depending on the thickness of leaf whorl) above the stem apex. *Agrobacterium* is injected at this position which results in the region between the developing leaves being flooded by *Agrobacterium* suspension.

After 5-6 days of injection, newly emerging leaves at the top show bleached regions which correspond to the regions that were transformed by *Agrobacterium* (see figure 3b). Hence the bleached regions can be used as a visual marker for identifying the portion of the leaves that are transformed by *Agrobacterium* in an injected plant.

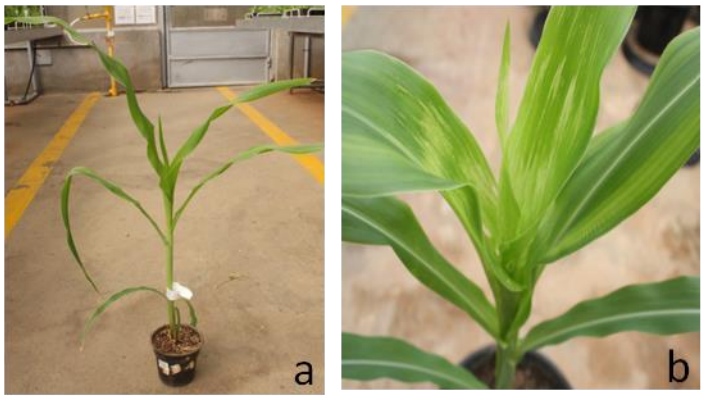

Fig. 3: *In planta* infection of corn leaf base (a) Stage and approximate position of in planta injection on a V5-V6 stage plant (b) The chlorotic regions of the infected zone on the emerging leaves.

GUS Assay:

Corn leaves are not permeable to X-gluc, the histochemical substrate of GUS enzyme. As X-gluc can penetrate only through injured surface, even stably transformed leaves stain only on the cut edges, (Fig. 4a). Hence, for detecting GUS activity on the surface, the leaf was injured as described in Fig.4b-c .

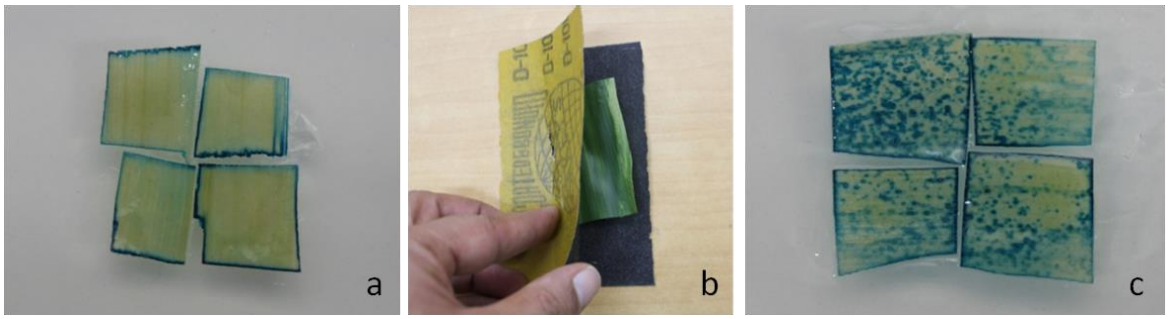

Fig. 4: (a) Stably transformed leaf (GUS gene) does not show staining on uninjured surface. (b) Creating pricks by pressing the leaf segments between sand paper to help X-gluc reach the GUS expressing cells on the surface of the leaf. (c) Staining on the surface when the transformed leaf was injured by sand paper before incubating in X-gluc. (Note: this experiment was performed with stably transformed leaf segments, but the results holds good for observing transient activity too).

GFP assay: The infectable zone (3-9 cms zone) of elongating leaf of corn was infected with *Agrobacterium* carrying GFP construct as described above. Three days after incubation, representative leaf tissue covering the length of the 3-9cms zone were fixed in 3.7% formaldehyde in PBS and washed three times in PBS before cryo-embedded and sectioned (20-25 um thickness). GFP was directly imaged under Zeiss 510META confocal microscope using 488 nm excitation and BP500-500 IR emission filters.

Results and discussion:

1. *Agrobacterium* mediated gene transfer to a large confluence of cells on the emerging leaves of corn:

In corn, a specific zone of emerging leaves at their base was found to be highly transformable as evidenced by strong reporter gene expression (GUS/GFP) within 3-days of *Agrobacterium* infection. This zone of *Agrobacterium* infection at the bottom of the monocot emerging leaf is referred hereafter in the rest of the article as 'infectable zone' (IZ). The emerging 8th or 9th leaves of corn at V6-V7 stage (Fig. 2) had larger infectable zone and hence further investigations were performed on these leaves. In these leaves, the highly transformable IZ region started from 3cms from the base of the leaf (point of insertion) and stretched to 9cms from the base (Fig.5).

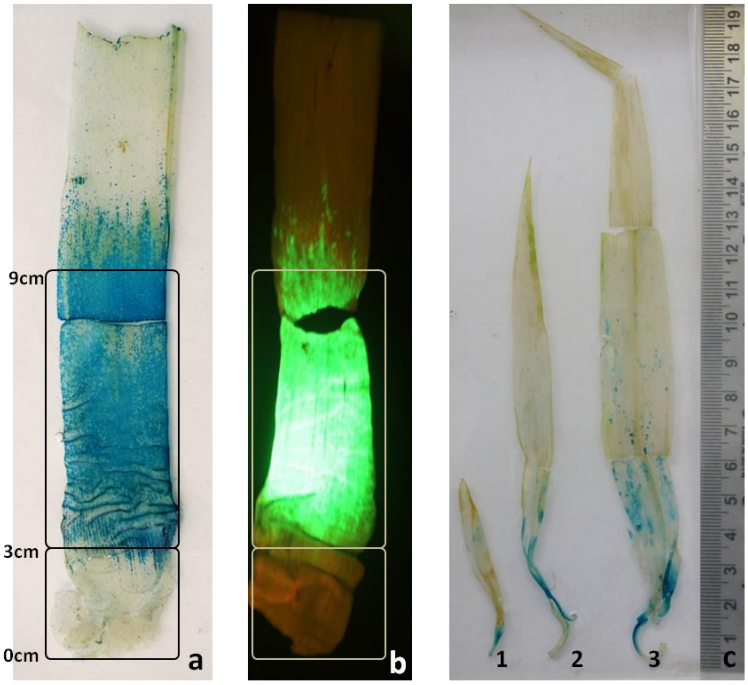

Fig. 5: Infection of elongating leaf base of corn with *Agrobacterium* carrying GUS / GFP construct. (a - b) 8th leaf (Only half the leaf blade after removing midrib and the other half have been shown). (c) 1st, 2nd and 3rd leaf respectively. Note, the leaves were harvested from plants at different stages, when the indicated leaf was emerging out of the leaf whorl.

Amazingly, every single cell in this wide region showed GFP expression which spread from the upper epidermis to the lower epidermis including stomatal cells, parenchymatous cells, the bundle sheath cells, phloem cells and pith cells making the leaf region indistinguishable from any completely transformed plant with respect to the reporter gene expression (Fig.6). This transformed nature of all cells was consistently observed in all the 10 representative cross sections across the IZ region. The size of the infectable zone (IZ) of the emerging leaf depended on the size of the leaf that it would develop into when it is fully mature. For instance, while the emerging 8^{th} or 9^{th} leaves had maximum infectable zones (Fig. 5a-b), the first 1 - 3 emergent leaves had smaller infectable bands (Fig. 5c). Also, the number of emerging leaves in a particular plant depends on the growth stage the plant is in. Corn plants older than V6 stage bear at least 3 emerging leaves while younger plants have less number of emergent leaves. The IZ extends over a length of 6 cms and spreads over the entire 5cm width of the leaf base (Fig.5). Assuming an average cell size of 20µ X 20µ X 20µ (see Fig.6), the IZ zone of emerging 8^{th} leaf would approximately consist of a remarkable 75 million transformed confluent cells (in an area of $6X10^4$µ length by $5X10^4$µ breadth by 200µ thickness of leaf).

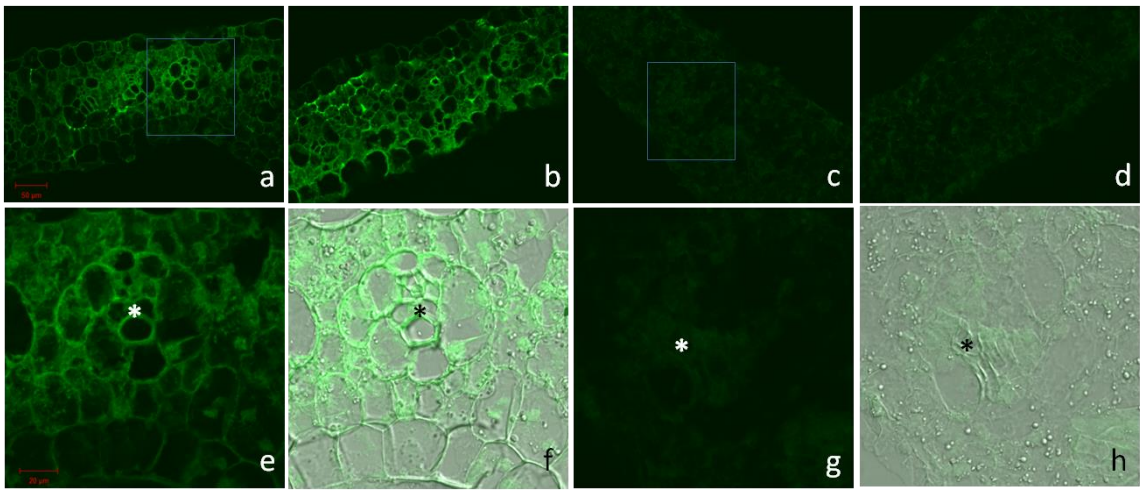

Fig. 6 : Confocal images in the 6cms infectable zone (IZ) of corn leaf base. (a, b, e, f) after infection with *Agrobacterium* carrying GFP construct. (c, d, g, h) negative controls. GFP channel (a-e, g) and merged images (f, h) with bright field of e and g, respectively.

(e and g) high magnification of boxed area in a and c, respectively, corresponding photographs of 6cms IZ zone infected with *Agrobacterium* carrying GUS construct. (b, d) Additional images of different leaf samples. Note the GFP expression in all the cells including cells within the vascular bundle (asterisks).

The different *Agrobacterium* strains tested (see Materials and Methods) did not show any difference in infectability. Further, though leaf bases of different genotypes of corn were infectable by Agrobacterium, the extent of infection slightly varied with genotype.

Agrobacterium infection was also high in plants when examined *in planta*, when it was injected in between the leaf whorls at the elongation region as described in materials and methods. However, with *in planta* infection, it relatively took longer time to detect the *Agrobacterium* infection as the infected elongated zone takes 5-6 days to mature and emerge out of the leaf whorl. Fortunately, the leaf patches which come into contact with *Agrobacterium* turn chlorotic before they emerge out of the leaf whorls (Fig. 3). These chlorotic patches when assayed for the expression of the transgene markers indicated extensive infection of leaves (Fig. 7). *In planta* infection confirms the ability of un-induced *Agrobacterium* to transfer gene to monocot cells under natural conditions. However, it is unlikely that this phenomenon happens in nature because of the extensive physical protection that these specific cell types are under in the monocot plants (discussed later). However, with in planta infected leaves, the transformed regions were interspersed by non-transformed regions which are unlike the in vitro transformed leaves (compare Fig. 5 and Fig. 7).

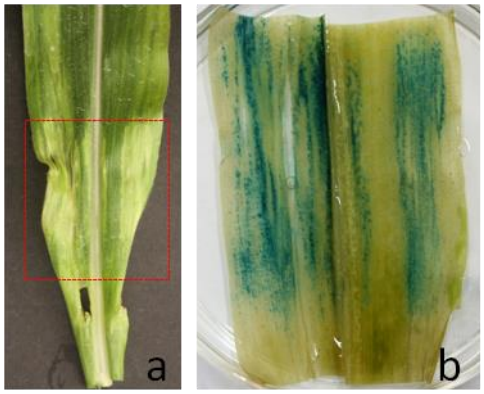

Fig. 7 : (a) Chlorotic patches on the leaf that emerged 6-days after *in planta* infection of *Agrobacterium* (containing GUS construct) expression (b) GUS expression in the boxed region shown in Fig. 7(a)

In planta transient expression systems for introducing *Agrobacterium* has been previously reported in switchgrass *(Panicum virgatum*) (VanderGheynst et al. 2008) and rice (Andrieu et al. 2012). In switch grass detached leaves were vortexed with a suspension of *Agrobacterium* followed by application of a low-pressure vacuum in presence of Silwet L-77. GUS expression could be detected in the leaf cells after 3 days incubation (VanderGheynst et al. 2008). In the leaves of rice (*Oryza sativa*), Ag*robacterium*-mediated transient gene expression has been reported, wherein the leaves of rice plants are mechanically wounded using 600-μm diameter needles. The wounded leaves were then incubated in a suspension of *Agrobacterium* containing the surfactant Silwet L-77 (Andrieu et al. 2012). However, in both the above reports, the infection is not as extensive as reported in the current report probably because the highly infectable leaf base was not exclusively employed.

It is a common experience that upon transformation either with *Agrobacterium* or particle bombardment, only a small proportion of target cells receive the DNA and only a small proportion of these cells stably integrate introduced DNA (Grant et al., 1991; Frank and Birch, 1991; Birch, 1997). In fact, this necessitates employment of selection agents during generation of transgenic plants. The remarkable competency of every single cell in the large IZ region to get transformed by *Agrobacterium* observed in the present investigation, to the best of our knowledge has not been previously documented either in dicot or monocot plant species.

Competency for *Agrobacterium* infection appears to be developmentally regulated:

It is interesting to note the clear zonation of competence in the elongating leaf for *Agrobacterium* infection, with the bottom non-infectable 0-3cms zone followed by highly infectable 3-9 cms zone quickly leading to the recalcitrant >9cms region in the 8^{th} or 9^{th} leaf at the time of emergence. In monocots, leaf differentiation proceeds basipetally

(from tip to base) in a highly regular and continuous manner (Li et al., 2010) and emerging leaves of corn, like in all monocot plants, have distinct dividing, elongating and differentiation zones in that order starting from the base. In a study of cell elongation of maize leaf, the cell lengths at various zones of emerging leaf have been documented previously (Muller et al., 2001). In the emerging 6^{th} leaf, the average cell length was reported to be minimum at the leaf insertion point (base) and increased slowly till the first 2cm but rapidly increased from that point before reaching a plateau at 8cm which marked the end of elongation zone (Muller et al., 2001). The 2-8 cms region of 6^{th} leaf where the cell length has been reported to rapidly increase and plateau roughly corresponds to the 3-9cms IZ zone of 8^{th} leaf in our study. The IZ region corresponding to the elongation zone is also supported by our observation of appearance of wrinkles / folds which form a particular pattern on the excised leaf after 3-days of initiation of culture. While the formation of wrinkles / folds in 0-3 cms zone was minimum, it was highest at 3cms (which is the beginning of IZ) gradually extending upwards and not detectable after 9cms (Fig.5 and Fig. 13). The formation of wrinkles of cultured leaf base, which were spread flat at the time of culture, is likely because of cell elongation during the course of *in vitro* incubation. The region at the 3cms, at the time of excision probably is expanding at a maximum rate relative to 0-3cms or region >9cms and hence produced the maximum amount of folds. Thus the infectable zone (3 - 9cms), where most of the cells get transformed, majorly overlaps the previously reported zone of elongation where most cells are less likely to be in the division state. These observations contradict the widely held belief that actively dividing cells is a necessity for *Agrobacterium* mediated gene transfer.

In fact the basal region where the division state is expected to be maximum was found to be completely recalcitrant under the conditions tested in the current investigation. This observed non-transformability of the basal 0-3cms region of elongating leaf could be because of non-availability of stomatal opening for the *Agrobacterium* to gain entry and/or insufficient intercellular space for *Agrobacterium* to move between the cells or the cells are inherently recalcitrant at this developmental state. The latter possibility is supported our observation that the basal 0-3 cms (which is not infectable on the day of

excision from the plant) became transformable by *Agrobacterium* when they were pre-cultured *in vitro* for 3-days (Fig.8). While in the same explants, the zone that was infectable by *Agrobacterium* on the day of excision became recalcitrant to infection after pre-cultured for the same number of days (Fig.8). It is likely that during the 3-days of culture, the cells in the 0-3 cms change from division state to elongation state concomitantly becoming competent for transformation. While the elongating cells in the 3-9cms changed from elongation state to mature state simultaneously becoming recalcitrant for *Agrobacterium* infection. The zonation pattern of infectability on freshly isolated explants and the pattern of changes of the same after pre-culture suggest that the cells progressively move from a state of recalcitrance to high competency and back to recalcitrance as they pass through the division, elongation and maturity stages respectively with the maximum competence for *Agrobacterium* infection during the elongation phase. Notably, these observations further contradict the widely held belief that actively dividing cells is a necessity for *Agrobacterium* mediated gene transfer which is probably true for dicot plant species but not necessarily true for infection of monocot plant tissues.

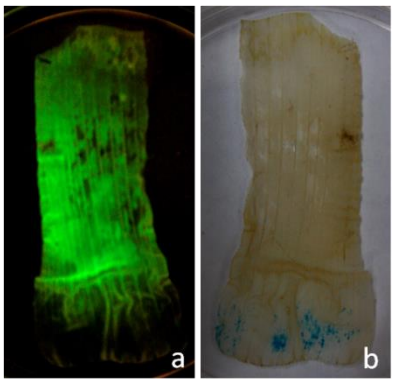

Fig.8: *In vitro* acquisition of competency for *Agrobacterium* transformation of 0-3 cms zone after 3-days of pre-culture *in vitro* (a) freshly isolated 0-9 cms leaf base infected with GFP construct carrying *Agrobacterium*. Note the absence of infection in the 0-3 cms zone (b) 3-day pre-cultured leaf infected with a GUS containing *Agrobacterium*. Note the development of competency for transformation in the 0-3 cms zone, while it is lost in the 3-9 cms zone.

3. Pre induction of *Vir* genes is not needed for the extensive transformation:

One of the critical factors necessary for *Agrobacterium* based transformation is the activation of *vir* genes by phenolic compounds released from the wounded plant cells (Li et al., 2000). It is generally believed that monocots are deficient in releasing these triggers of *Agrobacterium vir* gene (Li et al., 2000). Hence for cereal plant transformation, it is common practice to pre induce *vir* gene in *Agrobacterium*, before infection using phenolic compounds such as acetosyringone (Vasil, 2005). However there are some occasional reports of transformation of monocots without pre-induction of vir genes, like the reports on meristem transformation in sugarcane (Enriquez-Obregon et al., 1999) and pre-cultured immature embryo transformation in wheat (Cheng et al., 2003). In the present investigation, *Agrobacterium* grown overnight in LB medium without any *vir* inducer was found to be sufficient for the leaf base *in planta* transformation. Further wounding was not a requirement but actually was detrimental for *Agrobacterium* infection (Fig. S-1). The possibility of infection of large tracts of cells at the leaf base of corn without wounding or pre-induction of *vir* genes is in sharp contrast to the necessity of *vir* induced *Agrobacterium* for effective infection of immature embryos derived from the same corn genotype observed in our study (data not shown) which is also reported widely in the literature (Ji et al., 2013).

The above observation raises the question of the source of triggers involved in the *vir* gene induction in *Agrobacterium* when it infects IZ of emerging corn leaf and whether the availability of such compounds in this region (IZ) is the causal factor for its high infectability as compared to the >9cms or <3cms zones. Previously it has been reported that phenolic compounds are abundant in the emerging corn leaves especially near the elongation zone where they are condensed to form lignin as the cells mature (Maksimović, et al., 2008). It is possible that these free phenolics could induce *vir* genes of *Agrobacterium*. However, this explanation needs further experimental verification. As constitutively v*ir* active strains or pre-induced *Agrobacterium* did not extend the region of infection beyond the 9cm boundary (Fig. supplementary data), it can be

concluded that the phenolic compound availability is not the reason for the specific amenability of 3-9cms zone for *Agrobacterium* infection.

Many parallels can be seen between the results presented in the present work and the previously reported Agroinfection studies in corn (Schlappi, M. and Hohn, B., 1992). In both the cases pre-induction of vir genes are not needed for infection. Secondly, as in the present report, Agroinfection competency depended on growth stage of the explants. Further, undifferentiated meristem in the corn immature embryos was not competent and the competency was gained with the development of leaf initials (Schlappi, M. and Hohn, B., 1992).

Detergent treatment greatly enhances infection: Physical forces like creating a negative pressure employing vacuum or application of positive force with the help of needleless syringe are often employed in dicot plant species to push the Agrobacterium deep into the inner layers of plant tissue. However, these methods are known to be problematic in monocots because of several intrinsic structural features, such as extensive epidermal cuticular waxes, considerable silica content and the low volume of intercellular space in this group of plants (Krenek et al., 2015). In the above context, the mechanism that helps the movement of *Agrobacterium* to reach the innermost cell layers of leaf is intriguing.

The detergent Silwet L-77 could have a critical role in this *Agrobacterium* penetration of leaf as it had a dramatic effect on the extent of transformation of corn leaf bases (Fig.9a-b). Without Silwet L-77, infection was sporadic and mostly limited to regions near the wounds formed during handling and the cut edges (Fig. 9a). In contrast, Silwet L-77 treated explants showed infection throughout the leaf. Vacuum infiltration or application of positive pressure using a needleless syringe to infiltrate the *Agrobacterium* did not result in the extensive infection as that was observed with Silwet L-77 treated explants (data not shown).

Field and Bishop (1988) reported that Silwet L-77 promoted penetration of nearly all stomata by the foliar-applied solution in *Lolium perenne*, in addition to bringing about its complete and immediate spreading on the leaf surface. Further, Zidack et al., (1992) have demonstrated that Silwet L-77 is capable of facilitating stomatal penetration of bacterial cells on dicotyledonous leaves. Unlike other detergents Silwet L-77 has been reported to form smaller miscelles than the size of the stomatal pores which is hypothesized to help in better stomatal penetration than other detergents (Ref). In the present investigation, Silwet L-77 was the best among the 9 different detergents tested viz., Silwet L-77, Breakthrough, Ampholac YSH, Ethylan NS500, C6330, Ethylan1008, Phospholan PNP, AG6210, Phospholan PE65 (data not shown).

The corn leaf base appears water soaked within 3- mins of dipping in 0.01% Silwet L-77 solution (Fig. 2d). It is known that organosilicone detergents like Silwet L-77 bring about infiltration of the liquid containing the dissolved herbicide through stomatal pores (Stevens et al., 1991). This water soaked appearance of corn leaf base after Silwet L-77 treatment is indicative of penetration of liquid (perhaps along with *Agrobacterium*) into the intercellular regions. Curiously, the water soaked appearance is just up to the 0-9cms from the base and exactly coincides at the upper margin of the infectable zone (Fig.2d). Thus, the water soaked appearance brought about by Silwet L-77 and infectability are tightly linked as only the water soaked regions show the reporter gene expression, while the rest of upper regions strictly recalcitrant. Similar physical appearance of leaf has been reported in sunflower after Silwet L-77 treatment before *Agrobacterium* infection during transient expression (Manevalla et al., 2009), where too Silwet L-77 was indispensable. Thus Silwet L-77 perhaps not only thus helps bring *Agrobacterium* in close contact with the plant cells through wetting but also appears to aid penetration of *Agrobacterium* through stomata because of its chemical nature.

In the popular *Arabidopsis* floral dip method of transformation, buds are merely dipped in Silwet L-77 supplemented *Agrobacterium* culture, which has been found to be sufficient to transform their ovules (Desfeux, et al., 2000). Silwet L-77 eliminated the need of vacuum infiltration to help *Agrobacterium* to reach innermost target cells which was

previously found to be necessary for *in planta* transformation of Arabidopsis (Bechtold and Pelletier, 1998). The mechanism by which Silwet L-77 helps *Agrobacterium* to reach the megaspores, which are deeply hidden in the floral buds, with the 'floral dip method' of transformation, is still to be elucidated. It is possible that similar Silwet L-77 aided mechanisms are involved in *Agrobacterium* penetration needed for leaf base transformation of corn and the floral dip method of transformation in Arabidopsis.

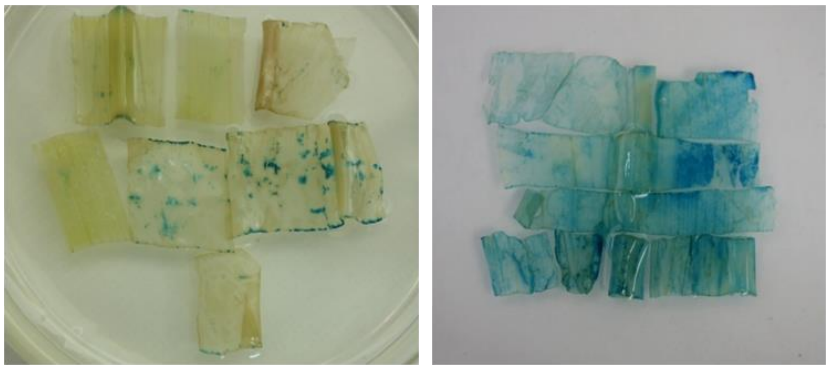

Fig. 9: Effect of Silwet L-77 on *Agrobacterium* infection of corn leaf base. (a) Leaf explants from the IZ zone infected without prior Silwet L-77 treatment (b) explants from the same zone, but infected after Silwet L-77 treatment as indicated in the materials and methods.

4. Efficient gene transfer to other corn organs which are known homologs of leaf:

The husk leaves, the glumes, the palea and lemma of male and female spikelets are known modifications of leaf in corn (Pozzi,et al., 2000). In the current work, all these leaf modifications too were found to be highly amenable for infection by *Agrobacterium* in their early developmental stages as was observed with emerging leaf base (Fig. 10). It is pertinent to note that the scutellum, a very common explant to produce transgenics in several monocot plants, is also a modified leaf and well known to be highly amenable for *Agrobacterium* transformation in its early stage of development. However, all these different leaf homologs lost their competency for *Agrobacterium* transformation when they were developmentally mature.

Stem and roots too have distinct division, elongation and maturation zones as monocot leaves. Hence we also tested the amenability of elongation zones of stem and root at different development stages in corn plant for *Agrobacterium* infection after similar treatments as with leaf base. Sporadic infection of *Agrobacterium* was observed at the elongating region of the stem. However, infection was limited to the surface layers and not visible in cross sections. In contrast the rachis of the developing tassel primordium, which are structurally similar to stem, was relatively more amenable for gene transfer (Fig.11). Roots were found to be harder to transform and rarely showed infection with only five of the 500 tested explants showing reporter gene activity (Fig.12). Roots have no stomata or any such natural openings as leaves and hence penetration of *Agrobacterium* to interior zones might be difficult. Moreover, developing roots are completely exposed even during early development hence are likely to have better defenses against microbial infection. This is unlike the leaf bases, which are physically well enclosed (Fig. S-4).

The commonality of the competency for *Agrobacterium* infection amongst leaf and their homologs, which is in complete contrast to that of roots, is possibly a reflection of a shared events amongst the former groups of organs during their development.

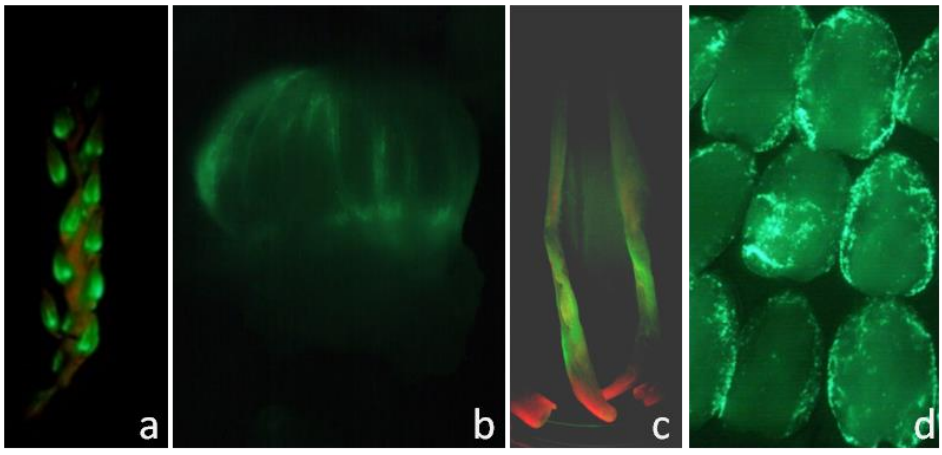

Fig. 10: Extensive un-induced *Agrobacterium* infection of leaf modifications (a) female spikelet (b) male spikelet (c) husk leaf and (d) scutellum of immature embryos (note the immature embryos were infected with *vir* induced *Agrobacterium*

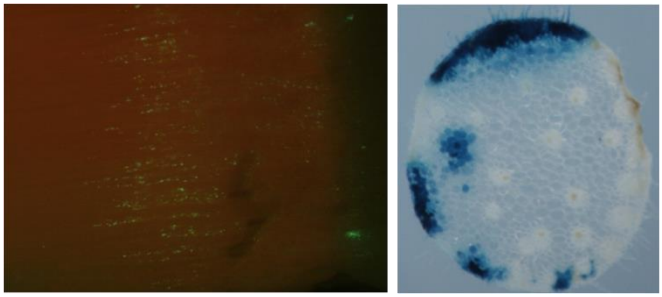

Fig.11: *Agrobacterium* infection of elongating stem (a) and rachis of the male immature inflorescence of corn (b)

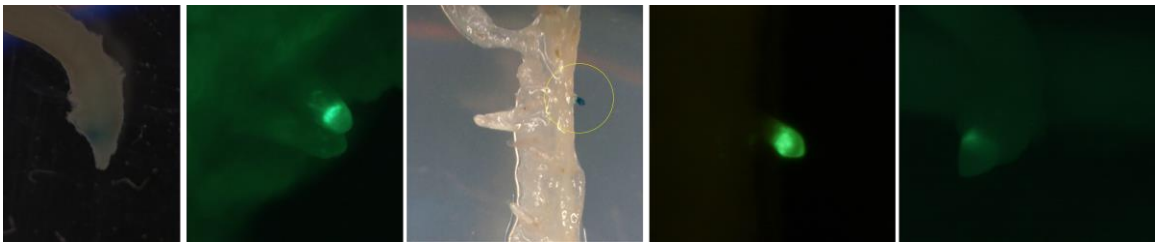

Fig. 12: Limited *Agrobacterium* infection of roots of corn. (Note: The five explants of the 500 explants tested, which showed reporter activity has been shown)

The phenomenon appears to be common in monocots: Other monocots like rice, wheat, banana and sugarcane showed extensive *Agrobacterium* infection at a similar zone at the base of their elongating leaf (Fig. 13). However, corn and sugarcane had relatively larger infectable zone as compared to the IZs of rice or wheat when tested under similar conditions of infection as in corn. This is understandable as the area of the elongating zone would be proportional to the size of the leaf that it elaborates into. Corn and sugarcane produce large leaves and hence are expected have relatively larger area of elongation zone and hence their infectable area would be more as compared to rice or wheat which produces relatively smaller leaves.

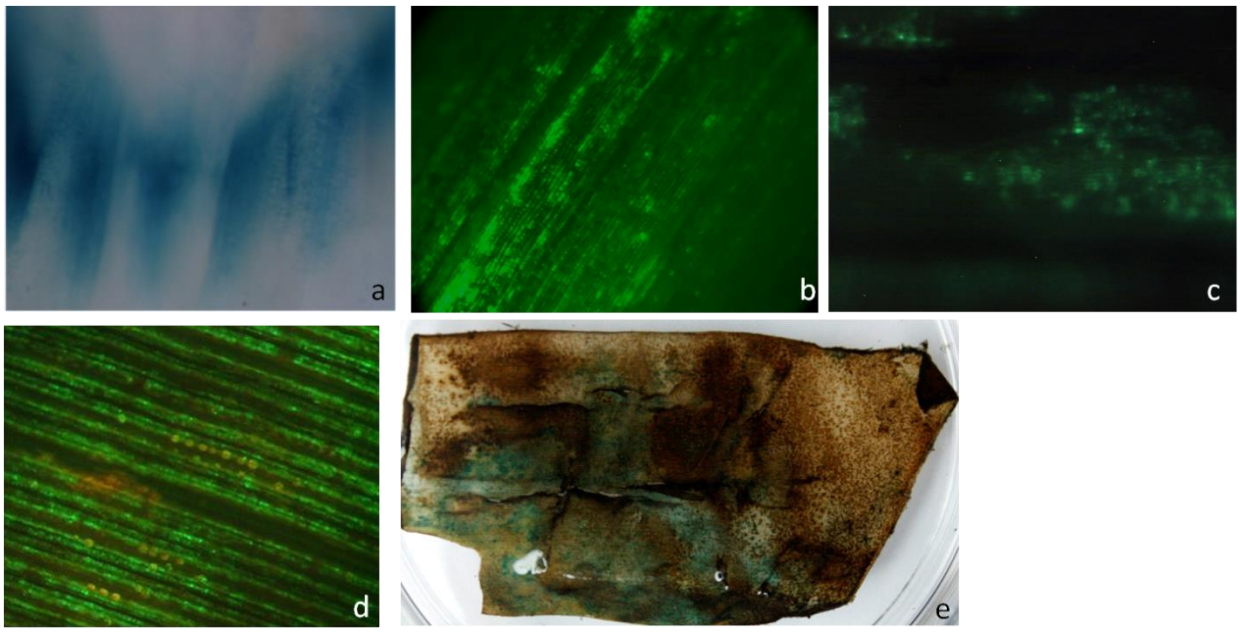

Fig. 13: Reporter gene expression leaf bases of different monocot plant species (a)GUS expression in rice (b) GFP expression in wheat (c) GFP expression in parlor palm (d) GFP in sugarcane and (e) GUS expression in banana. Note only the infectable zone is shown.

However leaves of none of the dicot plant species tested like soy, cotton, tobacco or okra screened at different development stages (from microscopic primordial stage to fully expanded stage) revealed any region where the cells were competent for *Agrobacterium* transformation comparable in extent to what was observed with monocot leaf base. Infection in these plant species was limited to injured areas occurring during handling of the explants (data not shown). Most often in these cases, the number of infected cells was countable under a microscope. Thus the high competence of the elongating leaf for *Agrobacterium* infection at their base seems to be a common phenomenon among monocots which is likely linked to their shared developmental events which are distinct from the events that occur during dicot leaf growth.

Competency of mature regions of emerging corn leaf for *Agrobacterium* infection:

As discussed above, Silwet L-77 could not bring about the water soaked appearance or the associated *Agrobacterium* infection in mature regions (>9cms from the base of 8th

leaf) of elongating corn leaf (Fig.2d). We used formation of water soaked appearance as a marker to identify treatments that help Agrobacterium infection on the mature regions of elongating leaf. Extensive water soaked regions could be obtained when 9-15cms mature regions of leaf were dipped in *Agrobacterium* culture with Silwet L-77 and vacuum infiltrated continuously for 15 mins. Three days after co-cultivation, GFP was detected spread over the entire leaf at regions corresponding to the water soaked patches that had formed following vacuum application (Fig.13). Though the extent of infection decreased as one moved up towards the tip, the GFP expression could be seen even at the most mature region (the tip of the leaf) (Fig.14). Agrobacterium infection in the >9cms zone could also be brought about by creating abrasions using a metal brush / sand paper while the explants were dipped in Silwet L-77 before infection. These mechanical injuries appeared to help create the water soaked regions on the day of infection and GFP expression could be seen in these regions after 3-days co-culture (Fig.S-3). These results clearly indicated that mature tissues of corn leaf are also competent for transformation provided the plant cells are made accessible to the *Agrobacterium* and this infection can happen without prior induction of *vir* genes. The above observations are similar to the previous report of Andrieu et al. (2012), who were able to obtain transient expression using *Agrobactreium* in leaf cells of rice after mechanical injury.

For expanding cells within the 3-9 cms zone leaf base, Silwet L-77 alone is sufficient for *Agrobacterium* to gain accessibility. However, for region >9 cms from the base, mechanical aids like prolonged vacuum or abrasions with a metal brush was needed along with Silwet L-77 for *Agrobacterium* to access the plant cells. Unlike dicots, grasses have been reported to have extensive interconnecting networks of phenylpropanoids which form primarily when cells stop expanding (Iiyama et al. 1990). It is possible that barriers like the network of phenylpropanoids acquired during maturity could possibly limit the access of *Agrobacterium* to the cells in the mature >9cm region. Further, prolonged *in vitro* incubation of the infected explants of the IZ region (supplementary Fig. 1) or the rest of the mature regions did not reveal any appearance of calli when observed under microscope. This ruled out the possibility of the treatments employed in the present investigation inducing cell divisions in the leaf which in turn facilitating

Agrobacterium infection. Taken together, the limiting factor for *Agrobacterium* transformation of monocot leaf cells appears to be a structural in nature which prevents the bacteria to reaching the plant cells rather than the competency of cells *per se* for transformation.

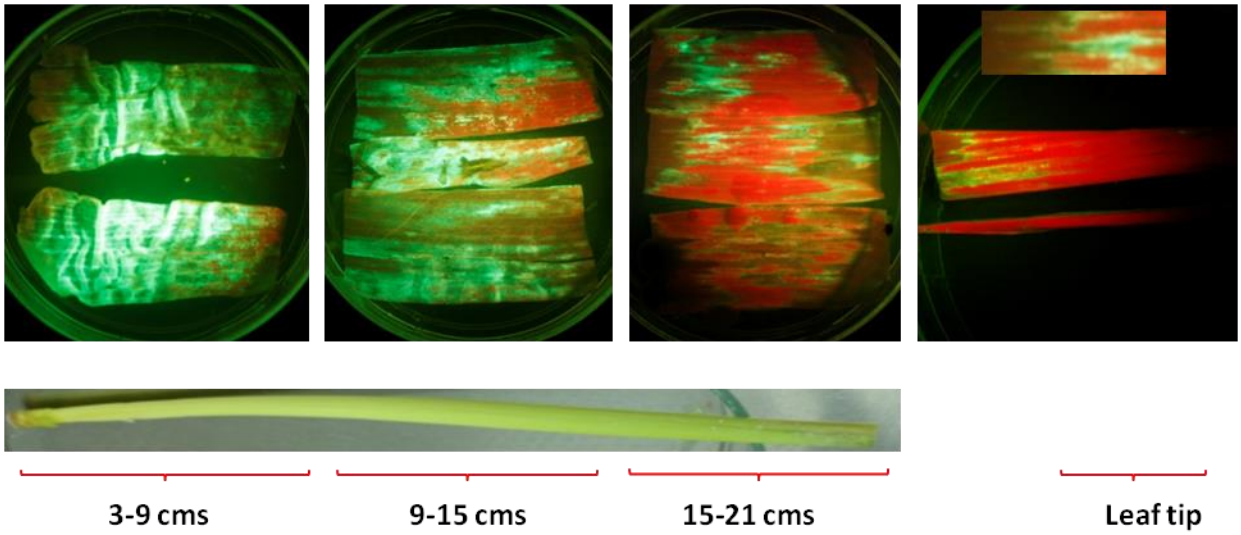

Fig.13: Competency of the complete elongating corn leaf for *Agrobacterium* infection with Silwet L-77 and vacuum as evidenced through GFP expression. The transformed cells fluoresced in green (because of GFP), the non-transformed cells fluoresced in red (because of their chlorophyll content) while the dead cells were transparent. Note that the transformed GFP expressing cells were sandwiched between the non-expressing dead transparent dead regions and non-transformed red fluorescent cells (Fig.9, inset). Also note the formation of wrinkles at the bottom of 3-9zone leaf explants and not in explants from upper mature regions.

5. Evidences suggest integration of transgene into chromosome:

In almost all the published work to date after ago-infiltration or co-cultivation, protein expression has been shown to be maintained only transiently, usually during the first 5–7 days which drops down drastically there after (Krenek et al., 2015). In the present investigation, there was no observable reduction in the GFP expression intensity when infected leaf explants were incubated for 4-weeks at room temperature and stored for

another 4-weeks at 4 ^{o}C (to control *Agrobacterium* overgrowth) (Fig. S-2). Further with *in planta* infection, GUS expression was seen stable for 7-weeks in the infected plants (beyond which observations were not made). The prolonged stability of transgene expression over many weeks after infection, suggested the possibility of chromosomal integration of the transgene. Taqman analysis for the transgene copy number in the isolated genomic DNA of the infected IZ zone showed the copy number to be greater than four. Southern hybridization proof for the integration of transgene was not possible, as the technique requires identical transgene integration in all the cells from which genomic DNA is extracted. However, this is not the case with the cells of the IZ as such large numbers of transformed cells are unlikely to arise through division within 3-days, especially in the context of the occurrence of this zone overlapping the elongation region. It is more likely that these millions of cells of IZ are the result of independent transformation events and hence are similar in containing the same transgene but different in their site of transgene integration.

Hence, we employed an alternate but indirect way of proving integration of the transgene to the genome employing an enhancer trap construct. Signal from an enhancer trap construct after transformation can only be obtained if the transgene lands next to an endogenous promoter in the genome in at least a few cells amongst the millions of cells that are transformed. Conversely, no signal is expected if the transformation does not involve integration of the transgene in the genome. In our experiments, many sparsely distributed GUS spots were observed in enhancer trap constructs infected explants (Fig.15a-b). On the other hand GUS expression was seen over the entire area of the explants infected with the positive control construct (Fig. 15c) and no GUS expression was observed in the leaf explant infected with a GFP construct (Fig. 15d). These results indicate the possibility of involvement of stable integration of transgene in the genome at the IR after *Agrobacterium* infection.

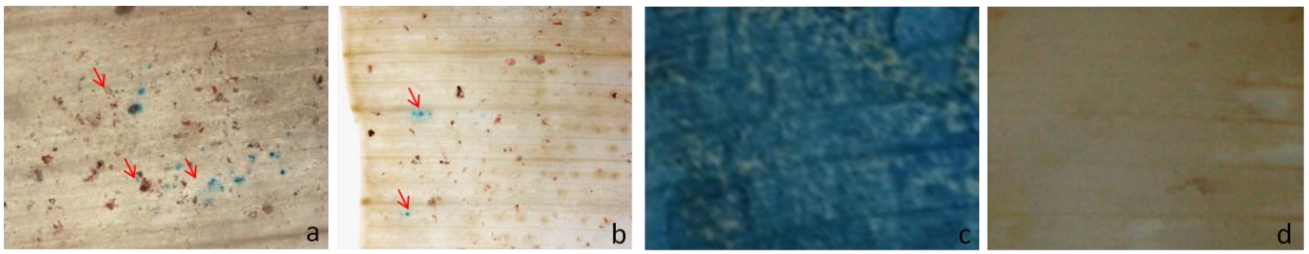

Fig. 15: (a-b) corn leaf base infected by enhancer trap construct (see materials and methods) and assayed for GUS expression (c) corn leaf base simultaneously infected with a construct with GUS gene under strong constitutive promoter e35S. Note the sparse blue spots in (a) and (b), indicating the probable cells where the transgene landed to next to an endogenous leaf active promoter (d) no blue spots were observed in leaf base infected with Agrobacterium containing GFP construct.

It is interesting to note the common thread running across the different *Agrobacterium* infectable tissues of monocots *viz*., their concealed existence in nature. For instance, the growing base of elongating corn leaf is exceptionally barricaded from the outside environment by the multiple whorls of tightly oppressed sheaths of earlier formed leaves (Fig. S-4). This physical protection of immature developing leaf bases by the older leaf sheaths is true for all the monocots studied in this report. Similarly the other highly infectable corn tissues *viz.*, developing glumes of florets, the immature embryos and the young husk leaves are also all deeply hidden when they are competent for transformation. In contrast, the roots (of monocots) or the leaves of dicots are continuously exposed to the environment right from their primordial state, which incidentally are comparatively less infectable by *Agrobacterium*. This protection of growing region is understandable as any damage to the leaf or stem meristem in monocots would result in irreparable damage, which is unlike the case of dicot plants where the meristem can form *de novo*. When they are in their protected confinement, the IZ cells of monocot leaves and leaf modifications are clearly vulnerable to pathogens as their structural barriers could be easily breached by Silwet alone. As the development proceeds, these IZ cells mature and possibly acquire strong physical barriers against pathogen attack, once they emerge out of the leaf whorls which make them recalcitrant even for *Agrobacterium* infection. On a

different note, the hidden nature of the highly *Agrobacterium* transformable monocot leaf tissue, probably is the reason why it went hitherto undetected.

In summary, we show that *Agrobacterium* can transform cells of developing leaf or their modifications over immensely large areas the extent of which, to the best of our knowledge, is previously not documented either in monocot or dicot plants. Our study opens up some of the intriguing aspects of *Agrobacterium* infection of monocot plants for further study like a) the mechanism of *Agrobacterium* movement into the innermost recesses of an intact leaf and the role of Silwet L-77 in the same (b) the source and the nature of the substances that induce *vir* genes of *Agrobacterium* when they infect unwounded monocot leaf base tissue (c) the particular developmental events which make the large but specific zone of elongating leaf so highly infectable while the cell layers immediately below and above remain recalcitrant.

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Supplementary data:

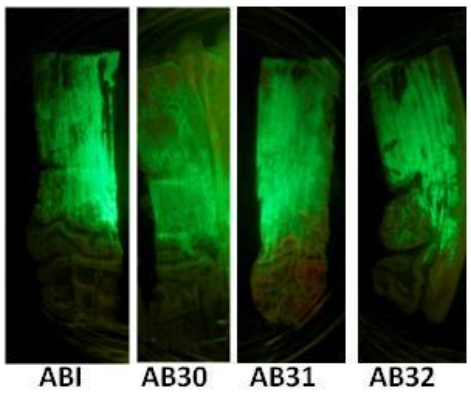

Fig. S-1: Effect of constitutively expressing vir *Agrobacterium* strains on the zone of infection. AB 30, AB31 and AB32 have constitutively expressing vir genes.

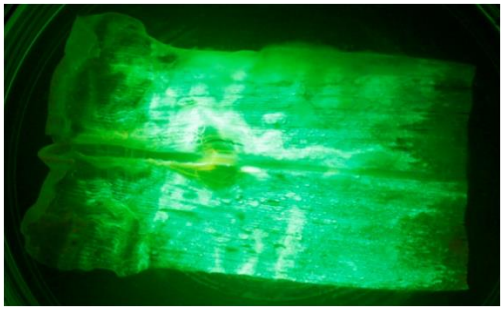

Fig. S-2: Transformed IZ stored for 8-weeks without any observable reduction in the extent or intensity in GFP expression (4 weeks at 23 ^{o}C and 2-weeks at 4 ^{o}C). Also note the absence of any calli formation after the prolonged incubation.

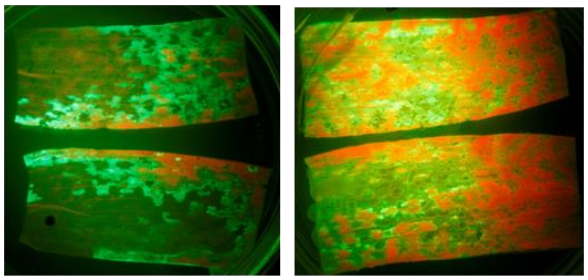

Fig. S-3: Infection of >9 cms zone after creating mechanical injury using a metal brush and pretreatment with Silwet L-77

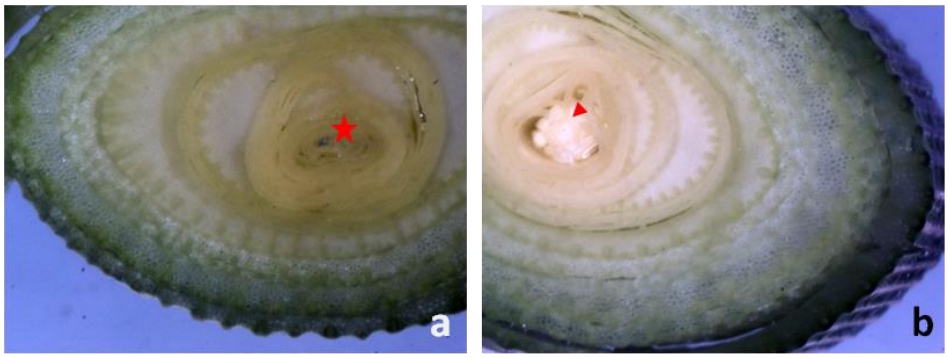

Fig. S-4: Ring fort like protection to the developing leaf base (shown asterisk) or the developing tassel inflorescence (arrow head) by the multiple whorls of leaf sheath of mature leaves in corn at V6 and V8 stage.