Selection of Pyramided Barley Advanced Lines for Stripe Rust, Leaf Rust and Crown Rust Diseases Using Molecular Markers

Resham B. Amgai1,2, Shreejan Pokharel1, Sumitra Pantha2, Atit Parajuli2, Sudeep Subedi2, Shambhu P. Dhital1
1Biotechnology Division, Nepal Agricultural Research Council, PO Box No. 121, Lalitpur, Nepal
2Agri Botany Division, Nepal Agricultural Research Council, Khumaltar, Lalitpur, Nepal

Abstract
Barley diseases are the major yield limiting factors for barley cultivation in Nepal. Stripe/Yellow rust (P. striformis f.sp. hordei and P. striformis f.sp. tritici), leaf rust (Puccinia coronata), and crown rust (P. coronata) are the major rust diseases in Nepal. Pyramiding resistance genes against all these rust diseases are possible through molecular marker assisted breeding. Sweden originated barley variety ‘Bonus’ is found resistant to stripe rust and having linked microsatellite markers for stripe rust and crown rust resistance. Similarly, Nepalese hull-less barley variety ‘Solu Uwa’ and Nepalese awn-less barley landrace NPGR Acc# 2478 have linked microsatellite markers for leaf rust resistance. Therefore, one polymorphic sequence tagged sites (STS) marker (ABC054) for stripe rust resistance, two polymorphic simple sequence repeats (SSR) markers (Bmac0144h and HVM049) for leaf rust and one polymorphic SSR marker (Bmg0006) for crown rust resistance were used to select the advanced barley lines (at F8 stage) from above parents. Field screening of stripe rust resistance was also conducted. Among 51 advanced and field disease resistance lines from Bonus/Solu Uwa cross, 10 pyramided lines for all three types of barley rust resistance were selected. Similarly, among 39 advanced and field disease resistance lines from Bonus/NPGR Acc#2478 cross, three pyramided lines were selected and advanced for further yield testing for general cultivation purpose. The chances of losing the desired gene are higher in late generation selection using molecular marker assisted selection (MAS), but the chances of getting agronomically superior varietal output is expected to increase.

Keywords: Rust, Pyramiding, Advanced lines, Barley, Marker Assisted Selection (MAS)

Introduction
Barley diseases are the major yield limiting factors in Nepal. Among many barley diseases, rust diseases are critical from the crop production view. Stripe/Yellow rust (caused by Puccinia striformis f.sp. hordei and P. striformis f.sp. tritici) is prevalent rust in the Nepalese barley field. Prasad et al. [1] also observed it as a major disease causing a problem in the Nepalese barley field. However, leaf rust (caused by P. coronata) can be observed in some warm barley cultivating areas. Crown rust of barley (caused by P. coronata) can be observed very sporadically only. Any barley variety having resistance gene for all three types of rust pathogen is highly sought in Nepalese barley breeding program.

Since, Nepalese barley germplasm has a high grain yielding capacity for hill and mountain regions of the country [2], adding rust resistance characteristics to them may improve their yield and stability. Selection, identification and incorporation of rust resistance genes is the only option for the development of rust resistance barley varieties for Nepal. Therefore, pyramiding major rust resistance genes for barley will be beneficial to farmers. Molecular markers are highly preferred for gene pyramiding program like this.

The sporadic nature of the crown rust occurrence in the Nepalese barley field and overlapping of leaf rust and stripe rust in the disease screening field further pushed molecular marker assisted selection (MAS) as the most viable option for gene pyramiding for rust resistance varietal development.

Materials and Methods
Parent and Advanced lines selection
A Swedish introduced variety ‘Bonus’ is the two-rowed stripe rust resistance barley variety for Nepal [2] and also have linked microsatellite markers for stripe rust and crown rust resistance. The polymorphic linked microsatellites are described in ‘identification of polymorphism in parents’ sub-heading. Similarly, Nepalese hull-less barley variety ‘Solu Uwa’ and Nepalese awn-less barley landrace ‘NPGR Acc# 2478’ has linked microsatellite markers for leaf rust resistance. Therefore, crosses between Bonus with Solu Uwa and Acc #2478 will have a lot of chances of having pyramided lines.

Use of marker assisted selection at early stage of barley breeding such as in F2 and F3 is practically not feasible in our context due to cost, time and manpower shortage.
every 0.5m. The length of the row was 1.5m. Resistance and

April) in 2017. Spacing between each row was 20 cm and

Two rows per line were sown at Khumatar, Lalitpur

Field Rust Evaluation

comparing to their parents.

So, we have selected 51 advanced barley lines at F8 stage

from ‘Bonus’ and ‘Solu Uwa’ crosses. Similarly, we have also

selected 39 advanced barley lines at F8 stage from

‘Bonus’ and ‘Acc#2478’ crosses to detect the pyramided

disease resistance. Similarly, they are forwarded to F8 based

on their superior agronomic characteristics comparing to their parents.

Field Rust Evaluation

Two rows per line were sown at Khumatar, Lalitpur
during normal barley growing season (November to April) in 2017. Spacing between each row was 20 cm and the length of the row was 1.5m. Resistance and susceptible parental lines were sown repeatedly after every 15 advanced lines. A susceptible landrace ‘Local Jau’ was used in two spreader rows around the disease

scoring at the heading stage for all three types of rust.

Identification of polymorphism among parents

A series of microsatellite markers linked with rust resistance genes were screened to identify the polymorphic markers among the parents. Sequence Tagged Sites (STS) marker ABG54, and Simple Sequence Repeats (SSR) markers Bmac144h, HVM49 and Bmag6 were found polymorphic among the parents (Table 2). These markers were used for the selection of advanced barley lines.

DNA extraction and PCR reaction

Modified CTAB method as described by Sul and Korban

[4] was used to extract the genomic DNA of selected barley advanced lines. PCR reaction mixture of 15 µl

| Parent | Field Stripe Rust | ABG54 (Stripe Rust QTL) | Bmac144h (Leaf Rust-R gene) | HVM049 (Leaf Rust-Rph19) | Bmag0006 (Crown Rust-Rpc1) |
|--------|-------------------|--------------------------|-----------------------------|--------------------------|-----------------------------|
| Bonus  | 0                 | 1                        | 0                           | 0                        | 1                           |
| Solu   | 105               | 0                        | 1                           | 1                        | 0                           |
| Uwa    | 606               | 0                        | 0                           | 1                        | 1                           |

Note: Number in bracket is the linked resistance gene. Acc# = NPGR Accession Number

Table 1. List of barley advanced breeding lines used for molecular marker assisted selection.

Table 2. Polymorphism observed in parental lines for different molecular markers and disease characteristics

©NJB, BSN
volume was prepared using 1.5 µl (1 µM) for each primer, 7.5 µl of PCR Master Mix (Promega Corporation, USA), 2.5 µl water and 2 µl (100ng) DNA template. This PCR mixture was amplified as per the following protocol. For Marker ABG54, Bmag6 and Bmac144h
Thirty cycles: denaturation 30 sec at 95°C, annealing 1 min with temperature as per Table 3 and extension 2 min at 72°C. For Marker HVM49
Touch down PCR protocol was followed as 18 cycles of 1 min denaturation at 94°C, 30 sec of touchdown protocol with decreasing 1°C per 2 cycle from 64°C until 55°C as annealing and 1 min at 72°C for the extension. This touchdown cycle was followed by another 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C. The final extension was 7 min at 72°C and the final holding is at 4°C.
The PCR products were separated in 2% agarose gel in 1XTAE buffer at 100V for one hour. Gels were stained with ethidium bromide (0.1 µg/ml) and visualized under UV rays. The presence of a particular band size (Table 3) was considered the presence of a particular linked gene.

### Results
We have observed stripe rust in susceptible parents; however, we had not observed leaf rust and crown rust in Khumaltar conditions (Table 2). This suggests that the use of MAS techniques is very essential for pyramiding any resistance gene that cannot be screened in field condition at that time. Many breeding lines showed the presence of one or more genes for the rust resistance based on the particular marker band (Figure 1-4). However, the pyramided lines were very limited than our expectation for both types of crosses (Table 4 and Table 5).
Discussion

Selection on the late stage of the breeding may lead to eroding many useful lines with the important genes that showed the neutral effect in the previous season of field disease screening. The leaf rust and crown rust could not be screened in Khumaltar condition for all previous seasons, which ultimately lead us a few lines with leaf rust and crown rust resistance along with stripe rust resistance. But, the agronomic characteristics of our selected lines are superior and always safe from ending with disease resistance but poor yielding varieties.

Due to the less polymorphism between the parent Bonus and Acc#2478; we can select the lines with pyramided stripe rust and leaf rust resistance linked markers only. The linked marker for crown rust resistance found in ‘Bonus’ is also found in Nepalese landrace ‘Acc#1478’ (Table 2). We identified linked markers for the leaf rust resistance gene in Nepalese local variety ‘Solu Uwa’ and landrace ‘Acc#1478’ which support our observation of barley field at Khumaltar and surroundings with negligible infection from leaf rust.

Higher leaf rust resistance in Nepalese barley is also supported by the observation of Tyrshkin [9] and Henderson [10]. Tyrshkin [9] also concluded that Nepalese barley germplasm NB-3002 has one dominant gene for leaf rust resistance.

| Line          | Field Stripe Rust | ABG054 | HVM049 | Bmac0144h | HVM049 | Bmag0006 |
|---------------|-------------------|--------|--------|-----------|--------|----------|
| Bonus         | 0                 | 1      | 0      | 1         | 0      | 1        |
| Solu Uwa      | 10S               | 0      | 1      | 1         | 1      | 0        |
| Bonus/Solu Uwa-30 | 0             | 1      | 0      | 1         | 1      | 1        |
| Bonus/Solu Uwa-33 | 0             | 1      | 0      | 1         | 1      | 1        |
| Bonus/Solu Uwa-45 | 0             | 1      | 0      | 1         | 1      | 1        |
| Bonus/Solu Uwa-48 | 0             | 1      | 0      | 1         | 1      | 1        |
| Bonus/Solu Uwa-60 | 0             | 1      | 1      | 1         | 1      | 1        |
| Bonus/Solu Uwa-63 | 0             | 1      | 1      | 1         | 1      | 1        |
| Bonus/Solu Uwa-81 | 0             | 1      | 1      | 0         | 1      | 1        |
| Bonus/Solu Uwa-90 | 0             | 1      | 1      | 1         | 1      | 1        |
| Bonus/Solu Uwa-135 | 0          | 1      | 1      | 1         | 1      | 1        |
| Bonus/Solu Uwa-138 | 0          | 1      | 0      | 1         | 1      | 1        |

Note: 1 = Present, 0 = Absent

Table 4. List of selected advanced breeding lines from crosses between Bonus and Solu Uwa with corresponding molecular marker polymorphism.

Note: 1 = Present, 0 = Absent

Table 5. List of selected advanced breeding lines from Bonus and Acc#2478 cross with corresponding molecular marker polymorphism.

Note: 1 = Present, 0 = Absent
Similarly, we also observed that hull-less parent (‘Solu Uwa’) is less stripe rust susceptible than the hulled parent (‘Acc#2478’) (Table 2) as Baniya et al. [11] already concluded for covered (hulled) barley and naked (hull-less) barley collection for Nepal.

Conclusion
Selection in an early generation for marker assisted selection (MAS) program is considered a thumb rule; however, in the condition where laboratory resource is poor and costly than growing crops in the field, late generation selection for pyramided lines using molecular techniques will be still competitive. On one hand, the chances of losing the desired gene (or marker) are high; but in another hand, the chances of getting agronomical superior varietal output will also increase by late use of MAS techniques since early generation selection on MAS largely depends on particular gene(marker rather than crop performance itself.

Author’s Contribution
RBA selected parents and made the crosses. SuP, AP, SS advanced the lines and maintain them. RBA, SuP did disease scoring. RBA, ShP, SS did DNA extraction, PCR and gel electrophoresis. RBA did data analysis, wrote and finalized the manuscript. All the authors read and approved the final manuscript.

Competing Interests
No competing interests were disclosed.

Funding
Part of this research is conducted under NG-NARC-Fund # 411.

Acknowledgements
Not Applicable.

Ethical Approval and Consent
Not Applicable.

References
1. Prasad RC, Karki CB, Sharma S. Pathological Report on Barley. Hill Crops Proceedings. National Hill Crops Research Program, Kabre Dolakha, Nepal; 1993. PP 52-78.
2. Riley KW, Singh KM. Diversity and stability of barley in Nepal. 1980 [cited 2020 Nov 5]. Available from: https://idl-bnc.idrc.dspacedirect.org/bitstream/handle/10625/6009/40369.pdf
3. Peterson RF, Campbell AB, Hannah AE. A diagrammatic scale for estimating rust intensity of leaves and stem of cereals. Can J Res Sci. 1948; 26:496-500. doi: https://doi.org/10.1139/cjr48-033
4. Sul IW, Korban SS. A highly efficient method for isolating genomic DNA from plant tissues. Plant Tiss. Cult. Biotech. 1996; 2: 113-116.
5. Rossi C. Testing the effectiveness of barley stripe rust resistance QTL detected in Mexico and the USA against a possible new race in Peru and mapping of genes conferring resistance to leaf rust and mildew in the same population [MS Thesis]. [Oregon]: Oregon State University; 2005. 91p.
6. Agrama HA, Dahleen L, Wentz M, Jin Y, Steffenson B: 2004. Molecular mapping of the crown rust resistance gene Rpc1 in Barley. Phytopathology. 2004; 94(8):858-861. doi: 10.1094/PHYTO.2004.94.8.858
7. Jafary H, Albertazi G, Marcel TC, Niks RE. High diversity of genes for non-host resistance of barley to heterologous rust fungi. Genetics. 2008; 178: 2327-2339. doi: 10.1534/genetics.107.077552
8. Park RF, Poulsen D, Barr AR, Cakir M, Moody DB, Raman H and Read BJ. Mapping genes for resistance to Puccinia hordei in barley. Australian J Agricultural Research. 2003; 54: 1323-1333. https://doi.org/10.1071/AR02244
9. Tyryshkin LG: Genetic control of effective leaf rust resistance in collection accessions of barley (Hordeum vulgare L). Russian Journal of Genetics 2009, 45 (3): 376-378. https://doi.org/10.1134/S1022795409030181
10. Henderson, MT. Studies of sources of resistance and inheritance of reaction to leaf rust (Puccinia anomala Rostr.) in barley [Ph.D. Thesis]. [Minneapolis]: University of Minnesota, Minneapolis, 1945.
11. Baniya BK, Riley RW, Dongol DMS, Sherchand KK. Characterization of Nepalese hill crop landraces (Barley, Buckwheat, Finger Millet, Grain Amaranth, Foxtail, Proso and Barnyard Millets). National Hill Crops Research Program, Nepal Agriculture Research Council, Dolakha, Nepal; 1992. PP 7-17.